

# **Impact of using chemical dispersants on the microbial response and formation of marine oil snow (MOS) in northeast Atlantic waters**



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Submitted for the Degree of Doctor of Philosophy

Heriot-Watt University

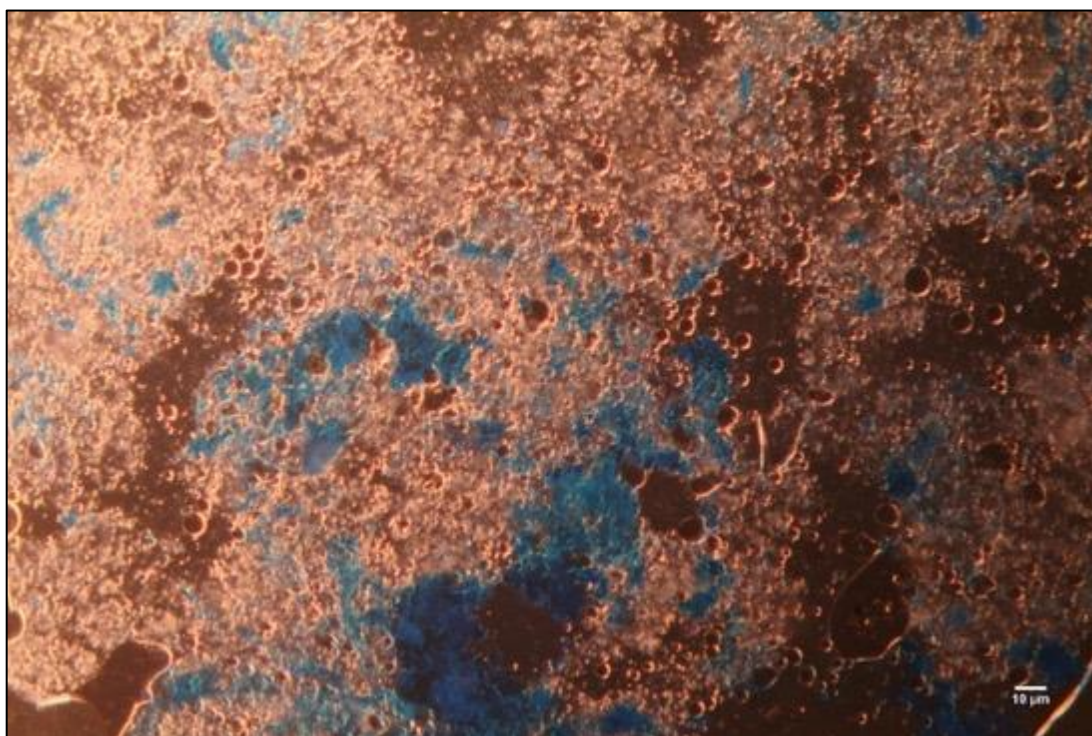
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## **Abstract**

Oil spills are one of the most catastrophic anthropogenic pollution events in the marine environment. With a rapid rise in economic development and energy demand around the world, marine petroleum exploitation and transportation has increased steadily, and with it also the risk for oil spills which often cause detrimental impacts to marine ecosystems. In the last years, an increase in oil activity in the northeast Atlantic has spurred interest in studying hydrocarbon-degrading bacteria in this region, particularly as these organisms are major actuators in the breakdown and ultimate fate of oil when it enters the sea. Of particular interest is the Faroe-Shetland Channel (FSC) where oil recovery is occurring and future exploration into its deep waters (beyond 500m depth) could increase the risk of major oil spills and that would be increasingly more challenging to combat. The FSC is notable for its predominance of the oil and gas sector, as well as being a unique hydrodynamic region defined by contrasting water mixing zones, variable physical conditions and large water masses that flow in opposite directions. It can thus be considered a region of interest to further research in trying to understand how autochthonous microbial populations would respond in the event of an oil spill in this region, identifying which oil-degrading species are most effective responders and degraders of the oil, and how certain factors, such as dispersant applications, might affect their response and activities. As reported during the Deepwater Horizon oil spill of 2010, the formation of marine oil snow (MOS), which was observed in unprecedented quantities floating on the sea surface within 2 weeks following the onset of the spill, predicting MOS formation in the event of a spill in the FSC is of significant interest. MOS formation, and its subsequent sedimentation, is one of the most important factors contributing to the vertical transportation of oil to the seafloor. To provide new insight into the fate of crude oil in the event of a spill in the FSC, this project firstly set to investigate the formation of MOS in waters of the FSC, the parameters influencing this process, and measure the oil biodegradation kinetics. Secondly, data collected from this first stage of investigation was then used and fed into models to understand the fate of oil entry in the FSC. This is an ongoing study in collaboration with a master student, so data won't be presented in this thesis. Thirdly, the effect(s) of different dispersants on the biodegradation of the oil and its effect on the microbial response in the FSC was determined. The findings from this project are expected to provide a new level of understanding on the fate of oil in the FSC, the factors that might influence this, and information to help contingency efforts for combatting future spills in this region.



**Star Blues**  
2<sup>nd</sup> Prize of the EPS Photography Competition

## **Dedication**

To my parents who have been working so hard all their life to ensure my education and progress during my whole career. This PhD has been possible because of the three of us, so congratulations. You are an example of perseverance and hard work and I would have never been able to get where I am without you, merci Maman, gracias José. I wish I will pass on the same childhood that I received.

To my sister, H  l  ne, for always showing admiration, interest (even if not understanding) and support during the whole process.

To my grandmothers Marie-France and Maria-Rosa who have taught me the real values of life and have always admired and supported me with anything I needed.

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A PhD is not an easy step in your life, and you get to realise how important are the people who surround you. I was lucky to find some great friends in Edinburgh: Afiq, Alice, Ana R, Danai, Fengjia, Johanne, Loris, Laurence E, Laurence DC, Margaux and Maria, thank you very much for all the dinners, surfing, brunches, nights out and the fun.

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## Research Thesis Submission

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Citation details	Suja, L. D., Summers, S., & Gutierrez, T. (2017). Role of EPS, dispersant and nutrients on the microbial response and MOS formation in the Subarctic Northeast Atlantic. <i>Frontiers in microbiology</i> , 8, 676.
Author 1	Laura Duran designed the work, interpreted it, produced all the data and wrote the manuscript.
Author 2	Stephen Summers helped in the data production.
Author 3	Tony Gutierrez helped in the experimental design, in the interpretation of data and edited the manuscript.
Signature:	
Date:	

Citation details	Duran Suja, L., Chen, X., Summers, S., Paterson, D. M., & Gutierrez, T. (2019). Chemical dispersant enhances microbial exopolymer (EPS) production and formation of marine oil/dispersant snow in surface waters of the subarctic northeast Atlantic. <i>Frontiers in Microbiology</i> , 10, 553.
Author 1	Laura Duran designed the work, interpreted it, produced all the data and wrote the manuscript.
Author 2	Cindy Chen did the EPS analysis.
Author 3	Stephen Summers helped in the data production.
Author 4	David Paterson helped editing the EPS analysis section.
Author 5	Tony Gutierrez helped in the experimental design, in the interpretation of data and edited the manuscript.
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Date:	



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## **List of Abbreviations**

**16S rDNA:** 16S ribosomal DNA gene  
**AB:** cationic copper phthalocyanine dye alcian blue  
**ANOSIM:** Analysis of Similarities  
**ANOVA:** Analysis of Variance  
**AO:** Acridine Orange  
**API gravity:** American Petroleum Institute gravity  
**APS:** Acidic Polysaccharides  
**CBBG:** amino acid-specific dye Coomassie Brilliant Blue G  
**CDOM:** Colored Dissolved Organic Matter  
**CEWAF:** Chemically Enhanced WAF (SW+O+Dispersant)  
**CEWAF+N:** CEWAF+ Nutrients  
**DAPI:** 4', 6-diamidino-2-phenylindole  
**DGGE:** Denaturing Gradient Gel Electrophoresis  
**DMSO:** Dimethyl Sulfoxide  
**DOC:** Dissolved Organic Carbon  
**DOM:** Dissolved Organic Matter  
**DWH:** Deep Water Horizon  
**EPA:** Environmental Protection Agency  
**EPS:** Extracellular Polymeric Substances  
**FIM:** Fair Isle-Munken  
**FITC:** Fluorescein isothiocyanate  
**FSC:** Faroe-Shetland Channel  
**FSCBW:** Faroe Shetland Channel Bottom Water  
**GCMS:** Gas Chromatography-Mass Spectrometry  
**HR-PCHO:** dilute-HCl-resistant polysaccharides  
**MDS:** Marine Dispersant Snow  
**MEIW:** Modified East Icelandic Water  
**MNAW:** Modified North Atlantic Water  
**MOS:** Marine Oil Snow

**MOSSFA:** Marine Oil Snow Sedimentation and Flocculent Accumulation  
**MRV:** Marine Research Vessel  
**MS:** Marine Snow  
**NAW:** North Atlantic Water  
**NMDS:** Non-metric Multidimensional Scaling  
**NRC:** National Research Council  
**NSAIW:** Norwegian Sea Arctic Intermediate Water  
**NSDW:** Norwegian Deep Sea Water  
**OHCB:** Obligate Hydrocarbonoclastic Bacteria  
**OMV:** Österreichische Mineralölverwaltung (*English: Austrian Mineral Oil Administration*)  
**OSAT:** Operational Science Advisory Team  
**OTU:** Operational Taxonomic Unit  
**PAH:** Polycyclic Aromatic Hydrocarbons  
**PCR:** Polymerase Chain Reaction  
**SNV:** Single Nucleotide Variants  
**SRA:** Sequence Read Archive  
**SW:** Sea Water  
**SW+D:** Sea Water+ Dispersant  
**SW+N:** Sea Water+ Nutrients  
**TCHO:** total dissolved carbohydrates  
**TEP:** Transparent Exopolymer Particles  
**WAF:** Water-Accommodated Fractions

## List of publications

**Publication 1:** Suja, L. D., Summers, S., & Gutierrez, T. (2017). Role of EPS, dispersant and nutrients on the microbial response and MOS formation in the subarctic northeast Atlantic. *Frontiers in Microbiology*, 8, 676. <https://doi.org/10.3389/fmicb.2017.00676>

**Publication 2:** Suja, L.D., Chen, X., Summers, S., Paterson, D. M., & Gutierrez, T. (2019). Chemical dispersant enhances microbial exopolymer (EPS) production and formation of marine oil/dispersant snow in surface waters of the subarctic northeast Atlantic. *Frontiers in Microbiology*, 10, 553.

**Publication 3:** Suja, L. D., Poulton, J., & Gutierrez, T. (IN PREP). Sources, Fate and Response to Oil Spills in the Faroe-Shetland Channel: A Microbiological Perspective. (for submission to *Marine Pollution Bulletin*).

**Publication 4:** Suja, L. D., Summers, S., & Gutierrez, T. (IN PREP). Effect of two different dispersants and seasonality on the bacterial community composition in the North Atlantic. (for submission to *PNAS* or *The ISME Journal*).



# **Chapter I: Introduction- Sources, Fate and Response to Oil Spills in the Faroe-Shetland Channel: A Microbiological Perspective**

## **1.1. Overview**

A description of the concept of marine oil snow (MOS), the factors influencing its formation, the microbial response to crude oil in the sea, and the fate of oil in the marine environment is reported in this literature review as an introduction to this thesis. A focus on the Faroe-Shetland Channel (FSC) is also presented here as it was the study site selected for investigation as part of this thesis, and also because of the current level of oil extraction activity in this region and because it is considered as a forefront for deep-water oil exploration. This chapter will form the foundation for the preparation of a manuscript review article for submission to the journal *Marine Pollution Bulletin* (see List of publications).

## **1.2. Introduction**

Oil spills are among the most detrimental anthropogenic pollution events in the marine environment. Due to the ability of oil to spread for many miles in seawater, their impact on ecosystems far exceeds the spills in terrestrial environments. With increased marine petroleum transportation, and its exploration in challenging environments (e.g. in deep-water provinces and the Arctic), the risk for disastrous crude oil spills has proportionally increased. The unpredictability of the behaviour of spilled oil and its ability to widely spread at sea has raised considerable concerns about the current experience and know-how in dealing with deep-sea spills. The Faroe Shetland Channel (FSC) is one such site facing the prospect of contamination from a major spill since oil exploration is expanding in its deep waters (>500m depth). Located in the northeast Atlantic between the Faroe and Shetland isles, it is a unique hydrodynamic region defined by variable physical conditions and large water masses flowing in opposite directions, and the formation of dynamic water mixing zones. The aim of this review is to provide an overview of the physical and geographical characteristics of the FSC, general hydrocarbon properties and its behaviour when it enters the marine ecosystem, and the potential paths of vertical and lateral flow of oil in the event of a deep-water spill in this region. The possible impacts of a spill in the FSC to local marine life will also be touched upon, with a focus more on the importance of microbial communities and the application of dispersants in the biodegradation of the different oil types under the prevailing environmental conditions. It is hoped that this review will provide a basic understanding on these various facets of oil spill response and fate of oil in the marine environment, and that from the research investigations conducted and reported in the chapters to follow,

that this will guide and instigate efforts aimed at improving oil-spill contingency plans for the FSC in the unfortunate event of a future oil spill in this region.

### **1.3. The Faroe-Shetland Channel (FSC)**

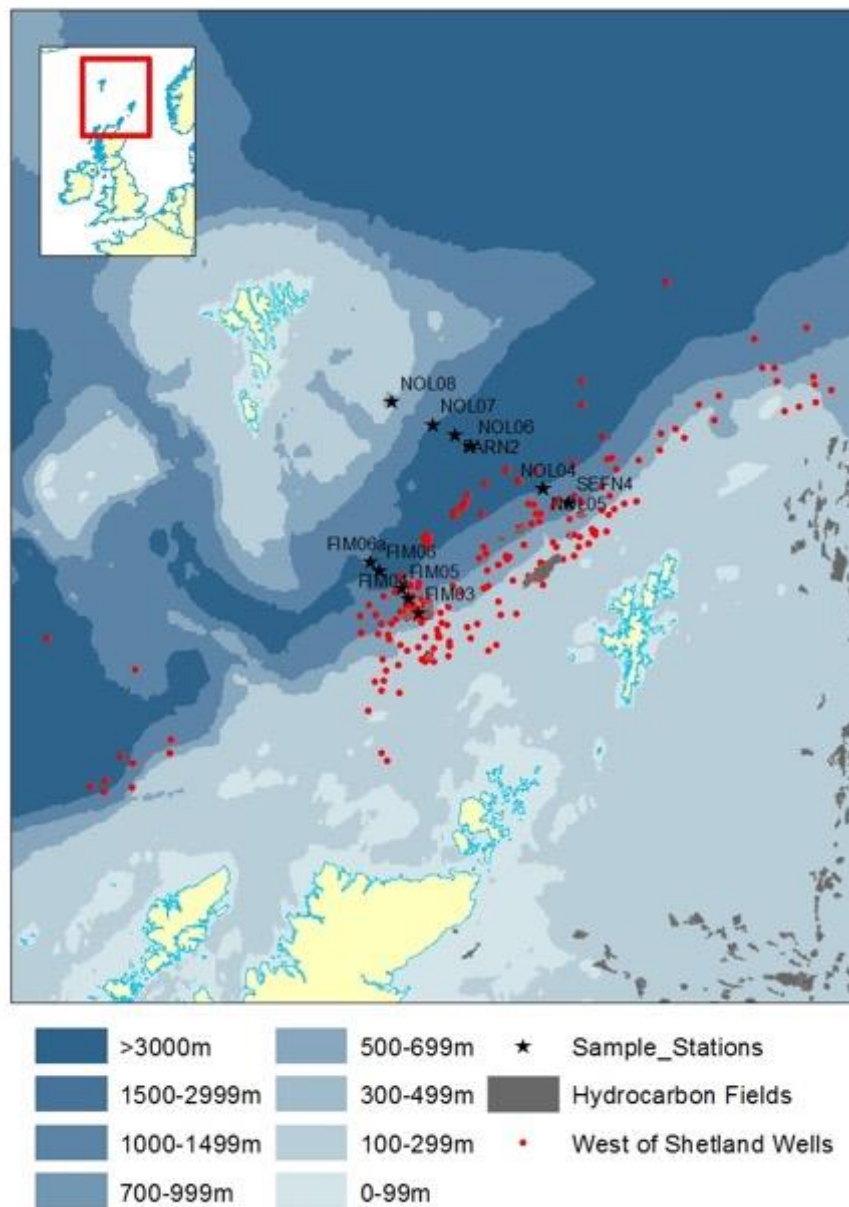
The FSC (Figure 1.1) is a rift basin located at 0-6°W, 60-63°N between the Faroe Islands (Faroese plateau) and the Shetland Islands (Scottish continental shelf). At the north, the channel is linked to the Norwegian Sea and can reach depths of 1600m. To the south, the channel is shallower reaching up to 850m and connecting to the Atlantic. Due to this configuration the channel is important in the exchange of water between the Atlantic and the Norwegian Basins. The FSC has been shown to be very atypical, as it has a wide variety of sedimentary habitats and an unusually dynamic temperature regime caused by the convergence of five contrasting water masses (described below). The thermocline region in the channel is at depth 350-650m and can exhibit hourly variations in temperature by up to 7°C (Turrell *et al.*, 1996; Berx, 2012). This habitat and environmental heterogeneity has a strong impact on the macrofaunal diversity and community composition, with temperature being the dominant variable dictating the diversity changes of the western region of the FSC, while sediment and organic matter being the dominant diversity-related variables in northern FSC regions (Hansen and Østerhus, 2000; Narayanaswamy *et al.*, 2010; Berx, 2012). The temperature fluctuates between 7°C in the northern regions to 11°C in the west regions.



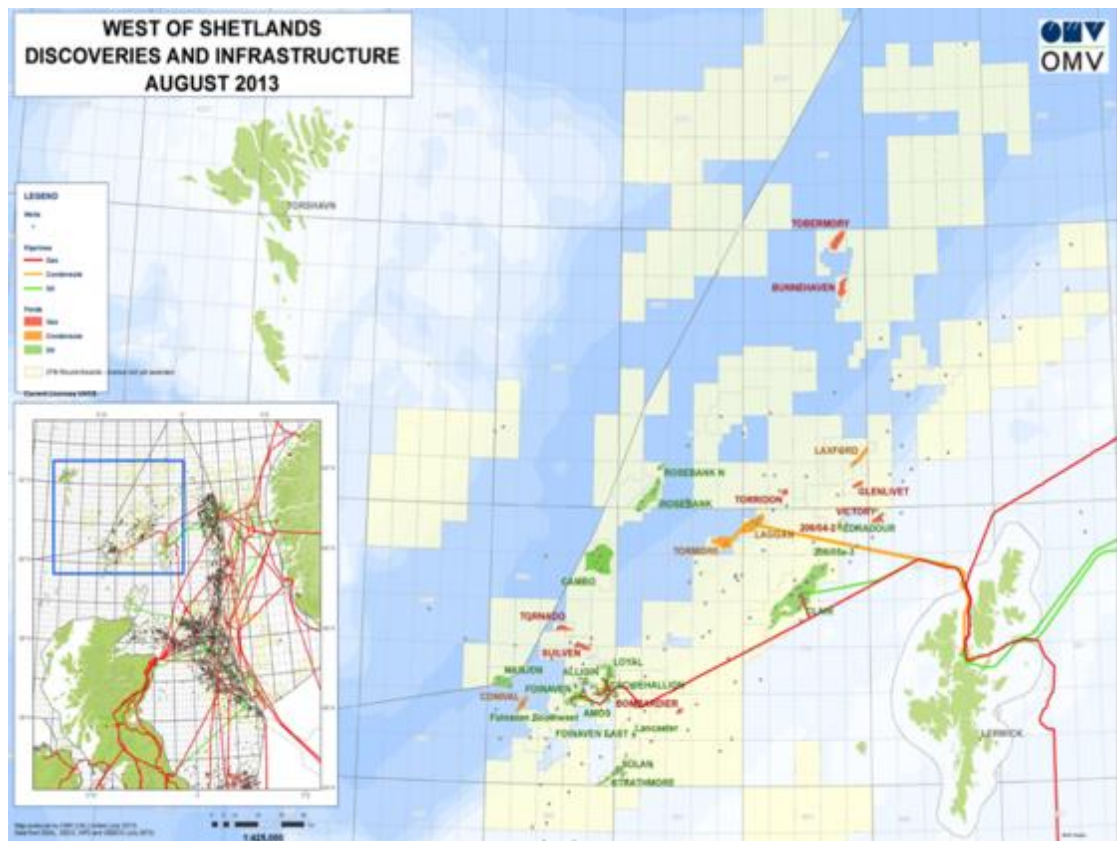
**Figure 1.1.** Map representing the location of the Faroe Shetland Channel (indicated by square).



In recent years, an upsurge of discoveries and development of deep water oil reserves in the FSC has led to the significant expansion of the oil and gas industry in the region (Figures 1.2 and 1.3) (Guevara and Lumley, 2012; Austin *et al.*, 2014). The major oil fields in the FSC (and year they started producing) include Clair (1997), Foinaven (1992), Schiehallion (1993), Loyal (1994), Rosebank/Lochnagar (2004), Tornado, Glenlivet, and Lancaster (2009), though there are other fields in the region.



**Figure 1.2.** Oil extraction and exploration activity in the FSC (Courtesy of Kate Gormley).



**Figure 1.3.** West of Shetland infrastructure map with significant oil field discoveries (Courtesy of OMV UK Ltd.).

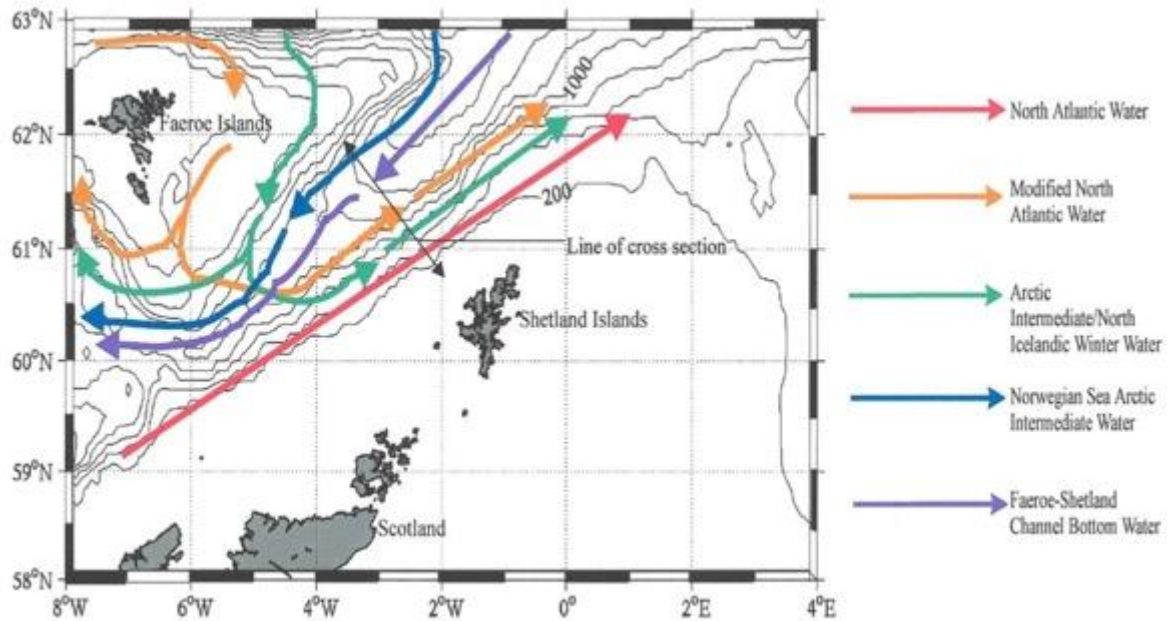
This development has also strongly raised the risk of oil pollution in the FSC, threatening the stability of local and adjacent ecosystems. Consequently, an interest in the response and biodegradation capabilities of autochthonous microbial communities, particularly oil-degrading bacteria and other organisms such as archaea, in the FSC has intensified in recent years.

The channel is characterised as a particularly dynamic mixing area of saltier (salinity) and warmer (temperature) Atlantic waters, which flow over colder, fresher (salinity) Arctic waters (Figures 1.4 and 1.5; Hansen and Østerhus, 2000). This encounter of different water masses presents a boundary region that is highly dynamic and where important changes in temperature and salinity occur. Each waterfront contains several water masses – two warm-water masses and three cold-water water masses (Figure 1.4; Turrell *et al.*, 1996).

### 1.3.1 Warm water masses

Hansen and Østerhus (2000) describe that there are the two Atlantic water masses: the North Atlantic Water (NAW) and the Modified North Atlantic Water (MNAW). The

NAW is the warmer and more saline surface water, confined primarily to the Scottish slope and exists inshore down to ~400m depth.



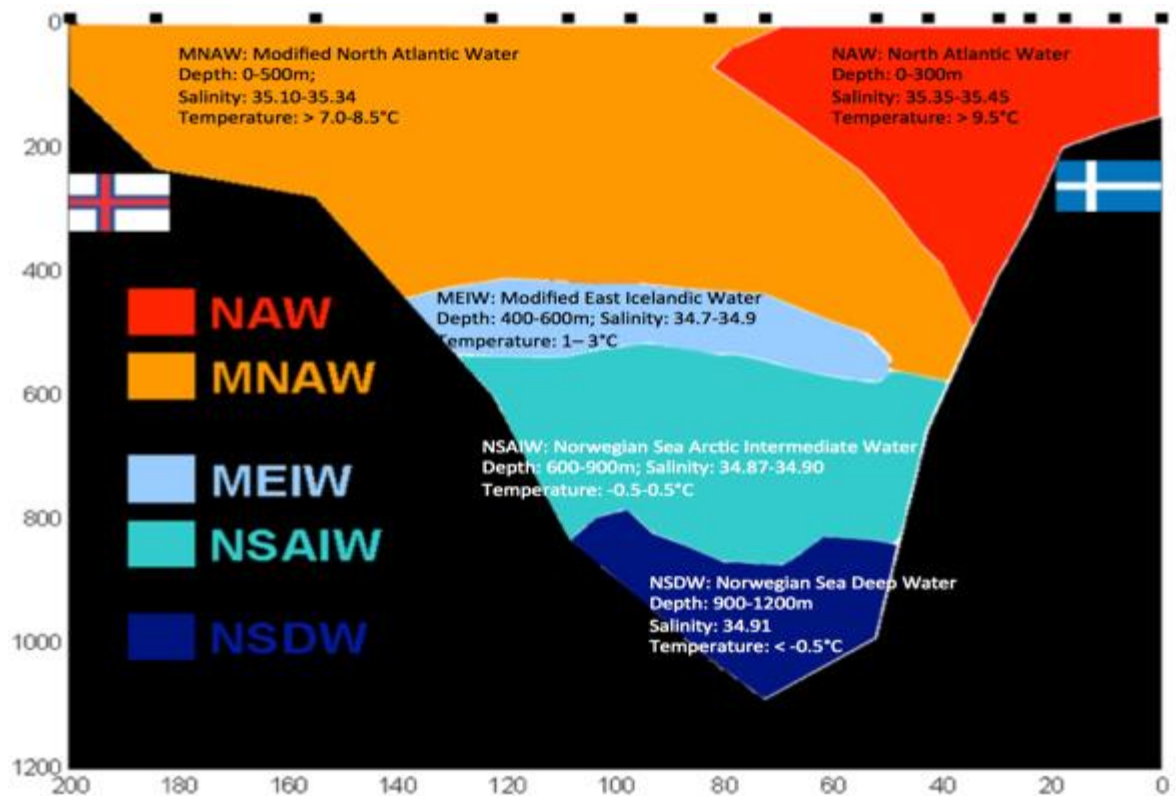
**Figure 1.4.** Schematic representation of the different water masses and their direction of flow in the FSC (adapted from Hughes *et al.*, 2006).

This water originates from the southern parts of the FSC around the Rockall Trough where the Atlantic Central water is more dominant. This water mass flows into the Norwegian Sea, as represented in Figures 1.4 and 1.5 (Turrell *et al.*, 1996). The MNAW originates from a branch of the North Atlantic Current and after some modifications (mixing with other waters) arrives in the FSC (Figures 1.4 and 1.5) from the northeast re-circulating within the channel to join the NAW flowing into the Norwegian Sea (Turrell *et al.*, 1996).

### 1.3.2 Cold water masses

There are three major cold water masses (Figures 1.4 and 1.5) - the Norwegian Sea Arctic Intermediate Water (NSAIW), the Modified East Icelandic Water (MEIW), and the Norwegian Sea Deep Water (NSDW) (Blindheim, 1990; Turrell *et al.*, 1996). The MEIW originates at the Faroe Island Ridge and settles within the 400-600m depth in the FSC beneath the MNAW. This water type is a mixture of Arctic and Atlantic water during winter convection on the north Icelandic Shelf, and therefore is not continuously present (nor easily defined) in FSC (Turrell *et al.*, 1996). The NSDW and the NSAIW are the FSC deep-waters, defined by lower temperatures and salinity. Both water types hardly

escape the Faroe Bank Channel and become re-circulated within the channel. The NSAIW is most likely created north of the Arctic front from the Icelandic and Greenland Sea waters, and has lowest salinity in the Channel.



**Figure 1.5.** Vertical water column cross-section of the Faroe-Shetland Basin showing the different water masses and their characteristics (Hughes and Turrell, 2006).

It flows in the FSC at depths 600-800m, above the more saline NSDW and below MNAW. The NSDW (also known as FSC Bottom Water (FSCBW)) lies below 800m and is primarily subsidised by the top layers of the Norwegian Sea's Deep Water. The FSCBW (Figures 1.5 and 1.6) outflows from the FSC by mixing with the surface waters and entering the North Atlantic through the Iceland Basin (Turrell *et al.*, 1996). The FSC is monitored seasonally by Marine Scotland Science (MSS), Aberdeen, through several cruises every year that track and collect data on the chemical and physical properties of the area. It is one of the most persistently monitored oceanic regions in the world, with systematic observations dating back over a century and continuing to this day (Berx *et al.*, 2013).

## **1.4. Petroleum in the marine environment**

### **1.4.1. Hydrocarbon properties**

Crude oil is a complex mixture of compounds, the composition of which varies based on the geological formation of the area from where the oil originated (NRC, 2003). The structure and physical properties of an oil are reflected in its composition, which generally consists of the following chemical groups (see Figure 1.6):

Firstly, the non-polar group (aliphatic or saturated hydrocarbons) includes *n*-alkanes, branched alkanes and cycloalkanes. These compounds are the easiest to biodegrade due to their structural simplicity (e.g. Kanaly and Harayama, 2000). Secondly, the aromatic compounds are another major constituent of crude oil, and include all aromatic ring-containing hydrocarbons, such as the non-polar polycyclic aromatic hydrocarbons (PAHs). PAHs contain two or more fused aromatic rings arranged in linear, angular or clustered form (Kerr *et al.*, 2001). The fused rings increase their degree of aromaticity, which increases their molecular stability (Hyne, 2012). Due to their poor solubility in aqueous liquids, PAHs are readily absorbed into fatty tissue of animals where they exhibit toxic, mutagenic and/or carcinogenic effects (e.g. Samanta *et al.*, 2002). PAHs represent a high priority pollutant in ecosystems as they can enter the environment from a number of sources, and they can make up as much as 60% of the composition of crude oil. For these reasons, they are considered of high concern to the environment and human health (Gutierrez, 2011; Fingas, 2016). Larger PAH variants may integrate other elements in their molecules, such as S, N and O, making their molecules more polar in composition (e.g. the asphaltenes). Their large bulky molecules also make them resilient to biodegradation.

Finally, crude oils also contain another type of aromatic polar component, but which is quite complex and referred to as the resins. These are mainly waxes that are largely responsible for oil adhesion (Fingas, 2016). There is also another group of hydrocarbons found in crude oils that are called olefins, which are unsaturated hydrocarbons (alkenes), the simpler members of which are used for the production of various plastics and synthetic fibres (Gaines and Shen, 1980). Depending on the ratio of these four components it is possible to consider oil as light (high in saturated and aromatic hydrocarbons) or heavy (higher in resins and asphaltenes).

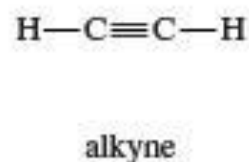
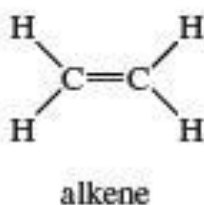
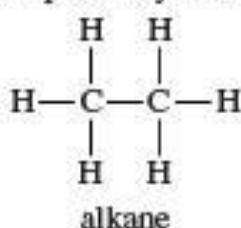
In the FSC, the most productive oil fields are Foinaven, Schiehallion and Clair, which are estimated to contain 250-500 million barrels of intermediate oil with an API (American Petroleum Institute) gravity of 24-28° (this corresponding to the inverse



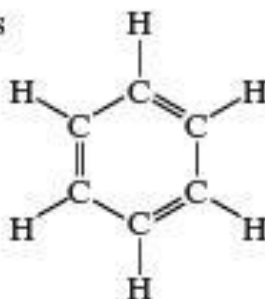
measure of a petroleum liquid's density relative to water's one; Scotchman *et al.*, 1998). The crude oil extracted in the Faroe Shetland Channel is a mixture of hydrocarbons under the form of liquid petroleum and natural gas originated from petroleum reservoirs (Middle Jurassic) under the North Sea Basin (Scotchman *et al.*, 1998). The most important crude oil is named Brent Crude (Brent Group) and it is defined as sweet light (intermediate light) crude oil due to its low density ("low") and low sulphur content (sweet, <0.42%) (New York Mercantile exchange, BBC, 2003). Results reported in Chapter IV of this thesis show GC-MS profiles of Schiellalion crude oil from the Faroe Shetland Channel, which shows the hydrocarbon profiles of the oil to be itself already quite weathered and biodegraded.

### Structures of representative hydrocarbons

#### aliphatic hydrocarbons



#### aromatic hydrocarbons



**Figure 1.6.** Structure composition of aliphatic and aromatic hydrocarbons. (Hydrocarbon, Encyclopaedia Britannica, inc., 2019).

#### 1.4.2. Fate of petroleum in the marine environment

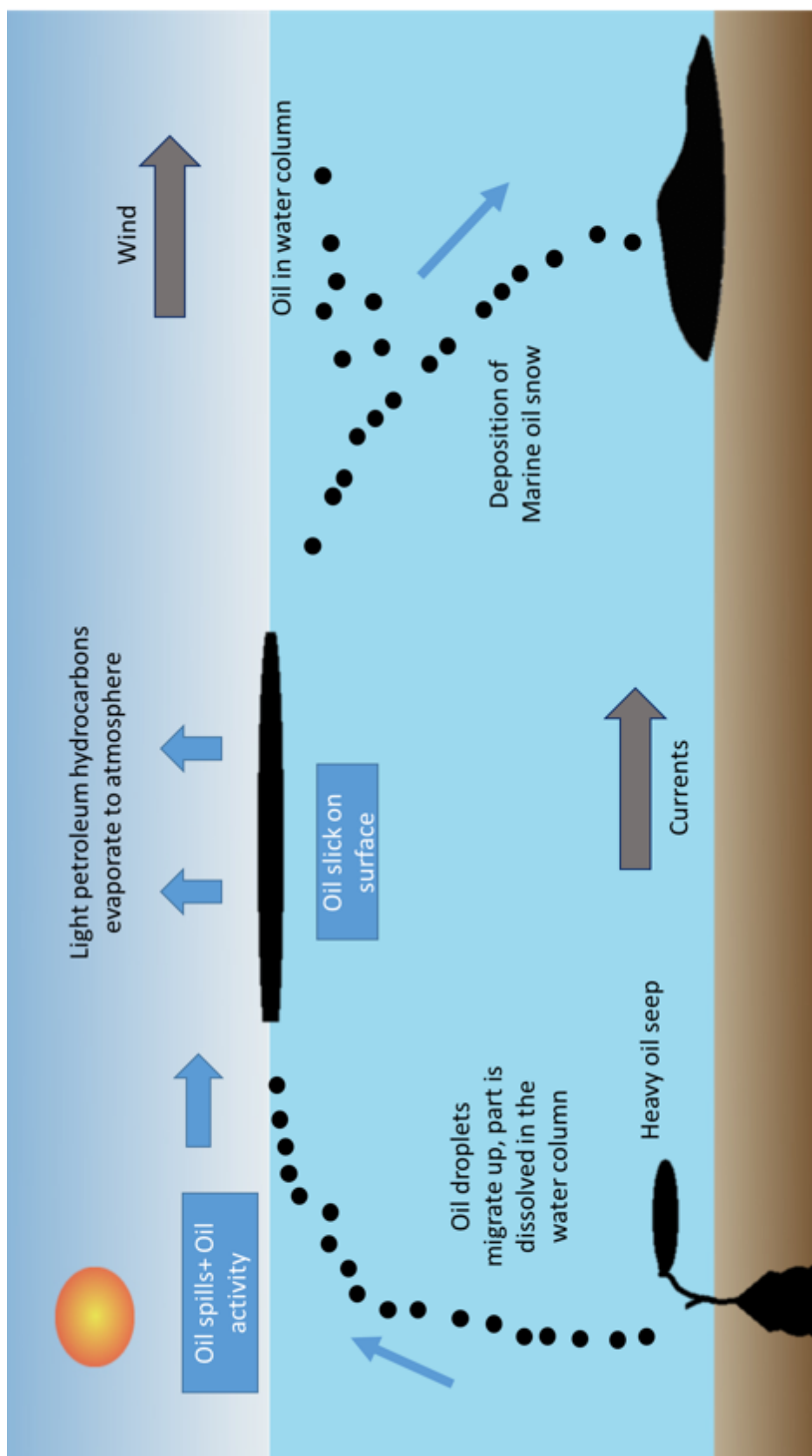
Upon its entry into the marine environment at the sea surface level (see Figure 1.7), oil will in the first instance form a slick where it will then be susceptible to various forms of physical, chemical and biological forms of weathering (Harayama *et al.*, 1999; NRC, 2003; Harayama and Hara, 2004). The type of oil, the prevailing environmental conditions (temperature, solar irradiation, mixing from winds and currents), and the

presence, abundance and efficiency of hydrocarbon-degrading microorganisms will all influence the rate and extent at which the oil is weathered or degraded (Harayama *et al.*, 1999; Harayama *et al.*, 2004).

At the onset of a spill, oil will spread over the sea surface due to the balance of three factors: gravity, viscosity and surface tension forces, while the composition of the oil will change due to weathering processes that encompass physical, chemical and biological forces (Wang and Fingas, 1995). Moreover, the physical and chemical properties of the oil, such as density and viscosity, will also contribute importantly to this process (NRC, 2003). Oil that enters the marine environment can form into droplets of different sizes as a result of dispersion and transformation processes led by environmental conditions (e.g. Dave *et al.*, 2011).

The dispersal of the oil droplets will then be affected by different factors (chemical, physical and biological) including advection, emulsification, sedimentation, dispersion, dissolution, evaporation, photo-oxidation and biodegradation (Huang *et al.*, 1983). Dispersion ocean factors (mainly weathering) and the physical-chemical properties of the oil are the main factors responsible for oil dispersal (Rasmussen, 1995; Socolofsky and Adams, 2002; Socolofsky and Adams, 2005).

Main factors involved are: advection - mainly carried by the wind and the waves affecting the oil droplet transport and size in the water column; depth - the degree of alteration of the oil would depend on depth (Corps, 2002); time - the amount of time that an oil droplet stays at the surface, determined by the balance between buoyancy and vertical diffusion rate, meaning that an oil droplet with a high buoyancy will remain longer on the sea surface and will thus be more strongly advected by waves, currents and wind (Wang *et al.*, 2008); size and velocity of oil droplets - oil can reach different velocities that control whether the oil droplets join the surface or form deep oil plumes as well as guide it in a specific direction (Kujanwinski *et al.*, 2011). Moreover, all these processes strongly depend on ocean density stratification, oil and dispersant chemical properties, the oil flow rate, the temperature at the source and upper water column, and the presence of gases mixed with the oil (Socolofsky and Adams, 2002; Socolofsky and Adams, 2003; Socolofsky and Adams, 2005).



**Figure 1.7.** General scheme showing the fate of oil in the marine ecosystem through natural and anthropogenic activities and suffers alterations in its physical-chemical properties by weathering, bio degradation and other processes.



All these factors make it very difficult to predict the fate and behaviour of the oil once it enters in the marine ecosystem. For instance, the consequences and the fate of the oil after the Deepwater Horizon (DWH) disaster in the Gulf of Mexico were much more important than expected. An oil plume was detected at around 1200 m depth at distances of up to 10 km from the wellhead (Camilli *et al.*, 2010) – the use of the dispersant Corexit to combat the spill could have influenced the formation of the plume (Kujawinski *et al.*, 2011). Its formation appeared to depend on several different factors, such as oil solubility, gas hydrate formation and the interface of oil in the multiphase flow (Johnsen and Rønningen, 2003).

A specific way for oil dispersion is vertical precipitation or deposition on the seafloor. Once oil enters in the marine ecosystem, oil can stick to DOM and other suspended particles in the ocean that lead to the formation of oil associated marine snow described in the next section 1.4.3 of this chapter. This snow will have a key role in oil transportation in the ocean and it is the key factor studied in this thesis.

### **1.4.3. Marine oil snow (MOS)**

Marine snow is composed of small organic and inorganic material, as well as prokaryotic (bacterial & archaeal) and phytoplankton cells (e.g. Simon *et al.*, 2002). It has a key role in the transport of organic material by gravitational settling to the seafloor (Lombard *et al.*, 2013), where it is then used as a food source by benthic communities (Newell *et al.*, 2005). It occurs at all depths all around the world and it is usually formed in the upper reaches of the water column, commonly in the euphotic zone where the bulk of primary productivity occurs (Daly *et al.*, 2016). The formation of marine snow is complex and different processes affect its formation, which include coagulation, flocculation and microbial activity (Alldredge and Silver, 1988). Particle size and its density are the principal factors determining the sinking speeds (De la Rocha *et al.*, 2008). Particles sinking slowly are easier to remove by microbial re-mineralization or grazing by marine organisms, and thus usually do not reach deeper depths. When marine snow forms, its downward flux is the principle process that defines the biological pump in the ocean, which exports carbon from the surface, and other elements, to deeper waters and the seafloor (Daly *et al.*, 2016).

During the DWH oil spill, oil-associated marine snow, called marine oil snow (MOS), of >1cm in size, was observed within two weeks from the onset of the spill, which

began on 11<sup>th</sup> of April 2010 (Fu *et al.*, 2014). Large quantities of MOS were observed in profuse quantities on the sea surface near and around the blowout (Passow *et al.*, 2012). Its formation was observed by scientists on the first research cruise that reached the spill site within 2 weeks from the onset of the spill on April 20 of 2010, and it sparked intense interest to understand the factors that triggered and influenced its genesis and evolution. Evidence of MOS formation has been reported for other major oil spills – namely the Ixtoc-I (Boehm and Fiest, 1980; Jernelöv and Lindén, 1981; Patton *et al.*, 1981) and *Tsesis* (Johansson *et al.*, 1980) oil spills. Like for marine snow, MOS is defined as oil-entrained mucilaginous flocs or particles ranging from >0.5 mm to 10s of centimetres in size, and composed of organic (e.g. exopolymeric) and inorganic (e.g. mineral) substances, microorganisms (e.g. bacterial and micro-algal cells), and other biogenic and inert components in seawater (Simon *et al.*, 2002; Passow *et al.*, 2012; Fu *et al.*, 2014; Daly *et al.*, 2016). Furthermore, Ziervogel *et al.* (2012) demonstrated that the suspended MOS particles acted as ‘hotspots’ for microbial oil-degrading activity, and Arnosti *et al.* (2016) showed MOS particles contained an associated bacterial community that was distinctly different from the free-living community in the surrounding seawater.

Whilst MOS formation may be a product of the interaction between suspended organic matter and oil (Fu *et al.*, 2014, Kleindienst *et al.*, 2015a), the underlying mechanism(s) in this process have yet to be fully understood. Various factors, such as hydrodynamic conditions, collision rate of suspended particles, particle coagulation and flocculation, and the interaction of particles with oil components/droplets, as well as with microorganisms and their produced exopolymers, are considered important in this process (Passow *et al.*, 2012; Daly *et al.*, 2016). However, some potential factors influencing MOS formation have been mentioned in the literature such as exopolysaccharides (EPS), nutrients or dispersant (e.g. Gutierrez *et al.*, 2013; Kleindienst *et al.*, 2015a).

It is well known that the biggest stocks of organic matter on the planet are in the oceans, usually under the form of dissolved organic matter (DOM) (Hansell and Carlson, 2001). The fact that DOM has a key role in MOS formation has been reported in several studies (e.g. Gutierrez *et al.*, 2013). It can have different functions and roles in chemical, biological and physical oceanography, and is the most important component fueling the microbial loop generating gasses and nutrients (Pomeroy, 1974; Azam *et al.*, 1983). The most important part of the DOM is produced and released by bacteria and eukaryotic

phytoplankton as extracellular polymeric substances (EPS) (Decho, 1990; Santschi *et al.*, 1999). A wide variety of microorganisms, particularly cyanobacteria (Decho *et al.*, 2005; Han *et al.*, 2014; Kawaguchi and Decho, 2002), bacteria (Grossart *et al.*, 2007; Kennedy and Sutherland, 1987; Thavasi *et al.*, 2011) and eukaryotic phytoplankton (Myklestad, 1977; Myklestad, 1995; Mishra and Jha, 2009; Raposo *et al.*, 2013) produce and secrete large quantities of EPS in the sea and this contributes a 10-25% to the dissolved organic carbon (DOC) in the global ocean water column (Verdugo, 1994; Aluwihare *et al.*, 1997). A number of studies have reported large quantities of EPS at the surface and deep-sea environments, including Antarctic marine waters (Mancuso Nichols *et al.*, 2004 and references therein). Marine bacteria can contribute to large quantities of EPS in the ocean (Azam, 1998), a large fraction of them under the form of glycoproteins (Long and Azam, 1996; Verdugo *et al.*, 2004). Moreover, other studies have also shown that uronic acids can give EPS the ability to interact with and increase the dissolution of hydrophobic organic chemicals, such as oil hydrocarbons (Janecka *et al.*, 2002; Gutierrez *et al.*, 2008, 2009). Amino acids and peptides are also often associated with marine bacterial EPS and can confer amphiphilic characteristics to these polymers and hence ability to interact with oils (Decho, 1990; Wolfaardt *et al.*, 1999; Gutierrez *et al.*, 2009). Laboratory experiments have been performed in plexiglass tanks maintained in constant rotation to simulate natural sea surface conditions and incubated under different parameters to investigate MOS formation (Shanks and Edmondson, 1989; Passow, 2016). Using cylindrical roller tanks containing seawater from two different sites, Shank and Edmondson (1989) reported the formation of marine snow. These aggregates appeared morphologically and chemically similar to the ones collected *in situ*, making this experimental setup a good tool for conducting MOS formation experiments in the laboratory. Following this idea, roller-bottle experiments were performed under similar sea surface conditions to those observed during the DWH spill, EPS produced by oil-degrading bacteria enriched in sea surface oil slicks was shown to trigger MOS formation (Gutierrez *et al.*, 2013), and similar results were observed with EPS produced by axenic cultures of eukaryotic phytoplankton (van Eenennaam *et al.*, 2016). This is studied further in Chapter III of this thesis where the application of dispersant seems to enhance EPS production.

Other factors that seem to have a potential role in MOS formation are dispersants and nutrients that have been shown to increase the quantity of MOS in the water column (Kleindienst *et al.*, 2015a; Chapter II of this thesis) as well as reducing the size of the

MOS flocs (Fu *et al.*, 2014). This will be further discussed in section 1.6 of this chapter. Furthermore, it has been suggested that increasing the residence time of MOS in the water column can enhance the biodegradation of the oil by microorganisms as well as decreasing its impact of the benthic ecosystem since the MOS will be biodegraded before reaching the seafloor (Daly *et al.*, 2016).

In other laboratory experiments, Passow (2016) investigated MOS formation with water from the Gulf of Mexico during the DWH spill site, herein with a focus on the effects of oil type and the presence of other external agents such as phytoplankton or dispersant. The subsequent behaviour and fate of the MOS was also analysed. Mucus-rich MOS was observed and its sinking behaviour reflected the importance of including the sedimentation of hydrocarbons via marine snow as a significant leave mechanism in the marine ecosystem. In a recent review article, Daly *et al.* (2016) described MOSSFA (Marine Oil Snow Sedimentation and Flocculent Accumulation) as representing the gravitational settling of MOS to the seafloor. MOSSFA is implicated as an important pathway for the transport and fate of almost 14% of the oil released during the DWH oil spill. However, detailed information related to the physical and chemical conditions influencing MOS formation, as well as what processes are involved in this process, is quite limited.

Only a few studies have reported the formation of normal marine snow aggregates with waters from the FSC-under no specific treatment (e.g. Chapters II, III and IV of this thesis; Summers *et al.*, 2018). Summers *et al.* (2018) described the formation of nano- and micro-plastic agglomerations (akin to marine snow or MOS) in waters collected from the FSC and in Chapters II, III and IV of this thesis and the experiments in Appendix B, marine snow formation in sea water treatments (only sea water) was observed. In this thesis, MOS formation is described in microcosms set up with surface seawater from the FSC for the first time (Chapter II of this thesis) and also during different seasons (Chapter IV of this thesis). In these studies, the effect of chemical dispersants and nutrient amendment on enhancing MOS formation is reported, as well as on inducing the synthesis of microbial-produced exopolysaccharides (EPS) which is suggested to enhance MOS formation and/or the biodegradation of the MOS-entrained oil. Interestingly, large MOS-like particles also formed in control incubations amended with the chemical dispersant but no oil. This work revealed then that the application of chemical dispersants and/or nutrients in the event of an oil spill in the FSC could lead to MOS formation and

enrichment of hydrocarbon-degrading bacteria whose abundance is concentrated on MOS particles. These findings are comparable to the ones described in other studies such as Passow *et al.* (2012) and Kleindienst *et al.* (2015a) in the Gulf of Mexico waters. Kleindienst *et al.* (2015a) showed similarly that MOS formation was enhanced by the addition of nutrients. However, MOS formation seemed suppressed by the amendment of the dispersant Corexit EC9500A.

For this thesis, other potential factors influencing MOS formation were contemplated such as depth, oxygen availability, light exposure and quorum sensing but those experiments stayed as preliminary experiments since not enough replicates for a proper study were used (see Appendix D of this thesis). Further research on this field would be very useful for designing better bio-remediation plans.

#### **1.4.4. Hydrocarbon effects on the marine environment**

Anthropogenic crude oil contamination in the oceans can come from different sources, such as urban and industrial wastewater, spills originating from tanker accidents, and leakages during oil and gas activity operations or marine transportation (NRC, 2003). Generally, the most important oil spill accidents have occurred in coastal zones and, often, have resulted in catastrophic and harmful consequences to marine ecosystems (Barron, 2012; White *et al.*, 2012; Almeda *et al.*, 2014 a,b,c). Several studies have analysed the weathering processes influencing the various hydrocarbons in the marine environment, and how this alters the toxicity and bioavailability of these compounds (Anderson *et al.*, 1974; Brodersen *et al.*, 1977; Abernethy *et al.*, 1986; Wolfe *et al.*, 1999; Hamdoun *et al.*, 2002; Kennedy and Farrell, 2005; Lee and Anderson, 2005; Hannam *et al.*, 2010; Sundt *et al.*, 2011). Other studies have investigated the impacts of petroleum to marine ecosystems, largely with a focus on coastal and shallow areas (e.g. Ko and Day, 2004; Nwilo and Badejo, 2006), which may largely be because of their accessibility. However, as oil exploration is expected to increase into new frontiers that are more extreme (e.g. deeper waters and the Arctic), there is a pressing need to do more research to investigate oil-spill impacts on these extreme environments (Jernelöv, 2010).

The DWH disaster, which occurred in deep waters off the De Soto canyon in the Gulf of Mexico (Zukunft, 2010) spurred intense interest from the scientific community to better understand deep-water oil impacts and the fate of the oil (Jernelöv, 2010; Norse and Amos, 2010). At the microbial level, several studies (Atlas and Hazen, 2011; Mason *et al.*, 2012; Gutierrez *et al.*, 2013; Yang *et al.*, 2015) reported a shift in microbial

communities within the water column in the area affected over the duration of the DWH spill. The microbial community of the Gulf of Mexico, initially similar and comparable to the one of the Atlantic and Pacific oceans, became temporarily dominated by oil-degrading bacteria after the oil spill (e.g. Gutierrez *et al.*, 2013). Other studies also reported microbial processes as key factors for the degradation of the oil in the plume—changes in the microbial community composition in the deep plume within one month of the spill (May 2010) were reported (Hazen *et al.*, 2010). Members of the order *Oceanospirillales* were reported to dominate the oil plume, and were in much less (almost undetectable) abundance in the uncontaminated surrounding waters at the same depth. A few months later, in June 2010, *Cycloclasticus* and *Colwellia* were reported to be the dominant members of the oil plume community (Valentine *et al.*, 2014; Yang *et al.*, 2016). Most studies focused on the microbial response in the deep oil plume (Camilli *et al.*, 2010; Hazen *et al.*, 2010; Valentine *et al.*, 2010; Kessler, 2011), whereas less focus was given to the surface oil slicks and their microbial evolution due to the oil (Edwards *et al.*, 2011). At the surface, oil was important in the formation of MOS, which was observed on the first research cruise (the Pelikan) and found floating on the sea surface in unprecedented copious quantities.

Not only are microbial communities affected by oil contamination. Studies reporting on the effects of oil to marine organisms have shown short and long-term effects in different invertebrate and vertebrate marine species (Jung *et al.*, 2012; Sammarco *et al.*, 2013), as well as decimation of plankton abundance due primarily to increased PAH concentrations (Jung *et al.*, 2012) and shifts in decapod communities were reported (Felder *et al.*, 2014). Long residence times of oil aggregates at the seafloor would strongly affect the benthic community of the area as well as the organisms feeding from this community (Daly *et al.*, 2016). Key organisms of the marine ecosystems such as corals and fishes have been described as quite vulnerable to oil pollution. It has been shown that oil contamination has detrimental effects on shallow and deep-water corals (e.g. Loya *et al.*, 1980; DeLeo *et al.*, 2016), especially on coral larvae settlement (e.g. Hartmann *et al.*, 2015). Moreover, after the DWH, some studies showed how crude oil, weathered crude oil and dispersants affect the early stages of fishes in different ways (e.g. Philibert *et al.*, 2019) although other authors such as Peterson *et al.* (2017) that studied the effects of oil contamination on coastal fishes did not find any evidence of the effect of the oil spill on those fishes.

One of the most affected marine fauna are seafloor associated communities (i.e., the benthic fauna). The planet is covered by marine sediments over 50% of its area and these play a very important role in ecosystem processes and ensure nutrient cycling (Widdicombe *et al.*, 2011). Sediments harbour a diversity of benthic fauna and microorganisms (Munn, 2011). Benthic ecosystem is usually sedentary and cannot easily escape from disturbance nor contamination so it stands as a highly vulnerable ecosystem to pollution (Fisher *et al.*, 2016). These organisms form part of an important stage of the marine food web and then can have an important impact on the whole marine ecosystem (Jones *et al.*, 1994). Pelagic and benthic individuals are strongly linked and depend on each other for feeding and reproduction purposes (van Eenennaam *et al.*, 2018). Due to the fact that MOSSFA participates in the vertical distribution of OM, any potential effect of it on the sediment and benthic habitats has risen a strong interest due to their potential long term effects on them (Stout *et al.*, 2017; van Eenennaam *et al.*, 2018). The benthic ecosystem can be affected in different ways by MOS precipitation and sedimentation. One effect could be due to the direct toxicity of the oil (Foekema *et al.*, 1996; Jewett *et al.*, 1999; Bhattacharyya *et al.*, 2003) and the dispersant. Indirectly, another effect of MOS reaching the seafloor is a reduction in oxygen availability in the sediments (Pelegrí and Blackburn, 1994) or changes in physicochemical properties of the environment mainly because of the microbial degradation (van Eenennaam *et al.*, 2018). Blackburn *et al.* (2014) described the effects of the oil from the DWH spill in the Gulf of Mexico on invertebrates, such as echinoderms, polychaetes, crustaceans, coral reefs and mollusks. A recent study from Passow *et al.* (2019) reports the incorporation of oil into diatoms aggregates that become heavier and sink faster reaching the benthic fauna and thus increasing the potential of a negative impact on it. While the effects of oil spills on invertebrate communities are often studied for a long enough time period, several studies show strong acute and long-term impacts of oil on these (e.g. Suchanek, 1993).

However, the potential effects of crude oil contamination to benthic fauna in the FSC remains largely unknown. A recent study in the FSC waters (Vad *et al.*, in press) has for the first time shown how shallow water sponges and their associated microbial communities respond to when exposed with oil and/or a chemical dispersant. *Halichondria panicea* sponge was found able to adapt and survive during exposure to crude oil and dispersant by altering its filtering behaviours over a short period of exposure. This, however, was one study that focused on only one sponge species in the FSC. Chapter II of this thesis showed that the application of dispersants in the FSC leads

to MOS formation in the event of an oil spill in this region, implying that during a MOSSFA event the benthic communities that receive the “shower” of MOS from the upperlying waters could potentially become exposed to toxic levels of the oil – albeit sponge communities like *Halichondria panicea* may not be susceptible to the toxic effects of the oil, as per the study of Vad *et al.* (in press). The FSC also contains other important benthic fauna, such as deep-sea corals (Roberts *et al.*, 2006), echinoderms, mollusks, polychaetes and other organisms (Jones *et al.*, 2007). It would be interesting in further studies to analyse the potential effects of oil hydrocarbons and/or MOS to other benthic fauna of the FSC. Based also on the strong currents that are prevalent in this region of the northeast Atlantic, it would be very interesting to model the trajectory of MOS and where it could potentially sediment on the seafloor in the event of a spill, as similar to modelling of the Macondo oil from the DWH spill (Main *et al.*, 2017).

### **1.5. Oil-degrading bacteria**

Crude oil naturally seeps into marine environments at an estimated rate of 700 million litres per year (NRC, 2003). Hence, microorganisms have over the course of evolution acquired the capacity to use hydrocarbons as a source of carbon and energy (Yakimov *et al.*, 2007; Atlas and Hazen, 2011, see Figure 1.8). Anthropogenic activities, however, have led to significant volumes of oil entering the marine environment each year (NRC, 2003). Of the total annual volume of oil that enters the seas and oceans, approximately half is derived from anthropogenic sources, whereas the other half is from natural seepage (Kvenvolden and Cooper, 2003).

The presence, properties and activity of oil-degrading microbial communities in oceans prevent the collapse of the ecosystem due to oil (Mishamandani *et al.*, 2014). Each class of components involves specific metabolic pathways for biodegradation (Fingas, 2011). Interestingly, the marine environment is one of the few places on earth where we find specialized oil-degrading bacteria – i.e. bacteria that use hydrocarbons almost exclusively as a sole source of carbon and energy (Head *et al.*, 2006). These so-called obligate hydrocarbonoclastic bacteria (OHCB) include members belonging to the genera *Alcanivorax*, *Cycloclasticus*, *Neptunomonas*, *Oleispira*, *Oleiphilus*, *Oleispira*, and others (Harayama *et al.*, 2004; Head *et al.*, 2006; Yakimov *et al.*, 2007). Some of these bacteria, such as *Alcanivorax spp.*, *Oleiphilus spp.* and *Thalassolituus spp.* use a wide range of branched- and/or straight-chain saturated hydrocarbons as their sole sources of carbon and energy. Others, such as *Cycloclasticus spp.*, use preferentially aromatic hydrocarbons, such as the PAHs. Head *et al.* (2006) reviewed that in different studies of



oil-impacted marine environments, after the addition of nutrients, these bacteria are rapidly and strongly selected for.

Generalist (non-obligate) hydrocarbonoclastic bacteria, that are also able to degrade hydrocarbons but that will also utilise other non-hydrocarbon substrates as a source of carbon and energy, include members belonging to the genera *Marinobacter*, *Vibrio*, *Marinomonas*, *Colwellia*, *Alteromonas*, *Halomonas*, *Pseudoalteromonas* and others (Harayama *et al.*, 1999). Upon entry of oil into the sea, up to 70-90% of the bacterial community can comprise oil-degrading taxa (Harayama *et al.*, 1999; Head *et al.*, 2006; Yakimov *et al.*, 2007), although the community profile, with respect to its diversity and abundance, will vary across different marine environments due to the prevailing biotic and abiotic factors, such as limitation of nutrients, temperature or salinity (Harayama *et al.*, 1999; Head *et al.*, 2006; Yakimov *et al.*, 2007).

Microorganisms play a fundamental role in the degradation of oil hydrocarbons and are at the heart of oil bioremediation processes. Few studies (e.g. Atlas and Bartha, 1992; Harayama *et al.*, 1999) have demonstrated that the available concentrations of some nutrients, such as nitrogen and phosphorus, in seawater are limiting factors for the growth of microorganisms that degrade hydrocarbons. Thus, the addition of these nutrients can influence positively the biodegradation of crude oil (Kleindienst *et al.*, 2015a; Chapters II, III and IV of this thesis). Generally, the small molecular-weight hydrocarbon compounds are easier to biodegrade than larger ones, whereas aromatic hydrocarbon molecules are degraded much slower compared to alkanes (Harayama *et al.*, 1999; Wang *et al.*, 2008). Some bacteria from different marine environments have been shown to degrade hydrocarbons in both oxic and anoxic environments (Leahy and Colwell, 1990), but the majority of bacteria will do so under strictly aerobic conditions. Anaerobic biodegradation is a significantly slower process (e.g. Haritash and Kaushik, 2009) and usually takes place in a sulfate-reducing environment (Ambrosoli *et al.*, 2005). In the aerobic biodegradation of hydrocarbons, the principal terminal electron acceptor is oxygen, with commonly the final end-products being carbon dioxide and water when complete mineralisation occurs. Oxygen might be important for a better chemical reaction of the oil degradation, but can become scarce at great ocean depths and when there is a surplus of oil carbon (e.g. Levin, 2002).

A single bacterial species is usually unable to do the whole process of oil biodegradation, and it is well recognised that a consortia of microorganisms participate

in the process and work together in the biodegradation process. This leads to a succession of different organisms along the oil biodegradation process (Head *et al.*, 2006). By following the microbial community dynamics, common patterns associated with biodegradation have been shown to commonly occur (Head *et al.*, 2006). Upon entry of crude oil into the sea, alkane-degrading taxa are the first to respond and become the dominant species until the bulk of saturated hydrocarbons have been degraded. A community of PAH-degrading taxa sequentially succeeds the alkane-degraders and becomes dominant (Head *et al.*, 2006).

MOS particles have in fact been reported as ‘hotspots’ for microbial activity (Ziervogel *et al.*, 2012) and to harbor bacterial communities enriched with oil-degrading and EPS-producing bacteria (Arnosti *et al.*, 2015; Kleindienst *et al.*, 2015a; Chapter II of this thesis). For example, in Chapter II of this thesis, the bacterial community present within individual MOS particles was analysed and the same for the surrounding seawater. The MOS-associated community profile was markedly different to the one of the seawater, the former of which was enriched with recognizable members of hydrocarbon-degrading taxa that included members of the genera *Alcanivorax*, *Pseudoalteromonas*, *Alteromonas* and *Psychrobacter* – taxa with reported EPS-producing qualities. Differential staining and microscopic observation of MOS particles revealed they are of glycoprotein composition and ‘hot spots’ for the aggregation of bacteria. This leads to the fact that marine bacteria have not only the ability to biodegrade oil but also of biosurfactant production. Crude oil might be degraded by specialised bacteria by hydrocarbon uptake, internal degradation or using extracellular enzymes (Gutierrez *et al.*, 2013) as well as emulsifier or surfactants to enhance the biodegradation (Harayama *et al.*, 1999). Emulsifiers and surfactants produced by the bacteria act as natural dispersants leading to the formation of small oil droplets and thus increasing the surface area of the oil for hydrocarbon attack (Salek and Gutierrez, 2016). These specialized bacteria have been considered as an important factor to control the fate of natural crude oil in marine ecosystem and are being studied (Head *et al.*, 2006; Gutierrez, 2011; Joye *et al.*, 2014; Silva *et al.*, 2014; Kleindienst *et al.*, 2015a; Daly *et al.*, 2016) to evaluate their capacities and limits in the case of anthropogenic hydrocarbon contamination (Hamdan and Fulmer, 2011). This might be a future tool for spill bioremediation and mitigation and a potential alternative to the use of synthetic dispersants.

## **1.6. Dispersants and their potential impacts**

At sea, dispersants are widely used as a response to an oil spill to enhance dispersion of the oil into droplets, the dissolution of the oil hydrocarbons, and which in turn increases the bioavailability of the hydrocarbons for degradation by oil-degrading microorganisms (Fingas, 2012; Prince and Butler, 2014; Kleindienst *et al.*, 2015a; de Almeida Couto *et al.*, 2016). Dispersants are formulations that are composed mainly of a solvent(s) and a chemical surfactant(s) (Kujawinski *et al.*, 2011) that act to reduce the surface/interfacial tension between the oil and the water (Ramachandran *et al.*, 2004; Greco *et al.*, 2006). Depending on the supplier, chemical dispersants can contain different types of surfactants (Hellgren *et al.*, 1999), but can also contain emulsifiers as part of these formulations; emulsifiers are a type of surfactant, though often of larger molecular-weight that act to also produce, but mainly stabilise, emulsions of oil and water (Ron and Rosemberg, 2002). After the addition of dispersants (see Figure 1.8), the oil mixes easily with the seawater surface enhancing its degradation and dissolution into the water column (Chapman *et al.*, 2007). The use of dispersants during oil spills is controversial since they can also be toxic to the marine ecosystem and extend the area of the oil spill that increases the potential impacts (Wolfe *et al.*, 1999; Epstein *et al.*, 2000; Edwards *et al.*, 2003; Ramachandran *et al.*, 2004; Couillard *et al.*, 2005; Greco *et al.*, 2006; Chapman *et al.*, 2007).

Currently, there are 23 US commercial dispersants approved by the Environmental Protection Agency (EPA) for combatting oil spills. Three of the most used dispersants worldwide are Corexit EC9500A, Corexit EC9500B and Corexit EC9527A (Kleindienst *et al.*, 2015b). Corexit EC9500A and EC9527A were the two used in large quantities (about 2.1 million gallons or 7.9 million liters) in deep water and at the sea surface (Lubchenco *et al.*, 2012; McNutt *et al.*, 2012), and which may have resulted in increased PAH concentrations in the water column in the DWH (Diercks *et al.*, 2010). Some studies concluded that, under specific conditions, the application of dispersants on surface oil spills can mitigate the impacts of the oil to coastal systems (e.g. Kujawinski *et al.*, 211). Nowadays, there are 18 commercial dispersants approved in the UK that are summarised in a list including name, nature and type of dispersant and what they are approved for (<https://www.gov.uk/government/publications/approved-oil-spill-treatment-products/approved-oil-spill-treatment-products>, MMO, GOV UK, 2019). Those dispersants would be the ones used in case of an oil spill happening in the FSC. Only few studies have studied the effect of those dispersants in the FSC. Perez Calderon

*et al.* (2018) studied the sediment bacterial community response under oil or/and dispersant addition in deep-waters of the FSC. The dispersant used in his study was Superdispersant 25 that is the most common one and comparable to Corexit 9500A (used in the Gulf of Mexico) and they observed significant changes in bacterial community composition after the addition of it to the sediments. The same dispersant, Superdispersant 25, was used in Chapter II of this thesis and it seemed to enhance MOS formation. Moreover, in Chapter III of this thesis, Superdispersant 25 is again used and its addition to the seawater seem to stimulate the bacterial community to secrete high quantities of EPS. Finally, in Chapter IV, two other UK approved dispersants, Slickgone NS and Slickgone EW, were studied. This last study shows that MOS forms under the presence of both dispersants and that the bacterial community composition can be different under the presence of one or the other dispersant. The nature of the dispersant seem to determine the bacterial community composition as well as the efficiency in oil biodegradation.

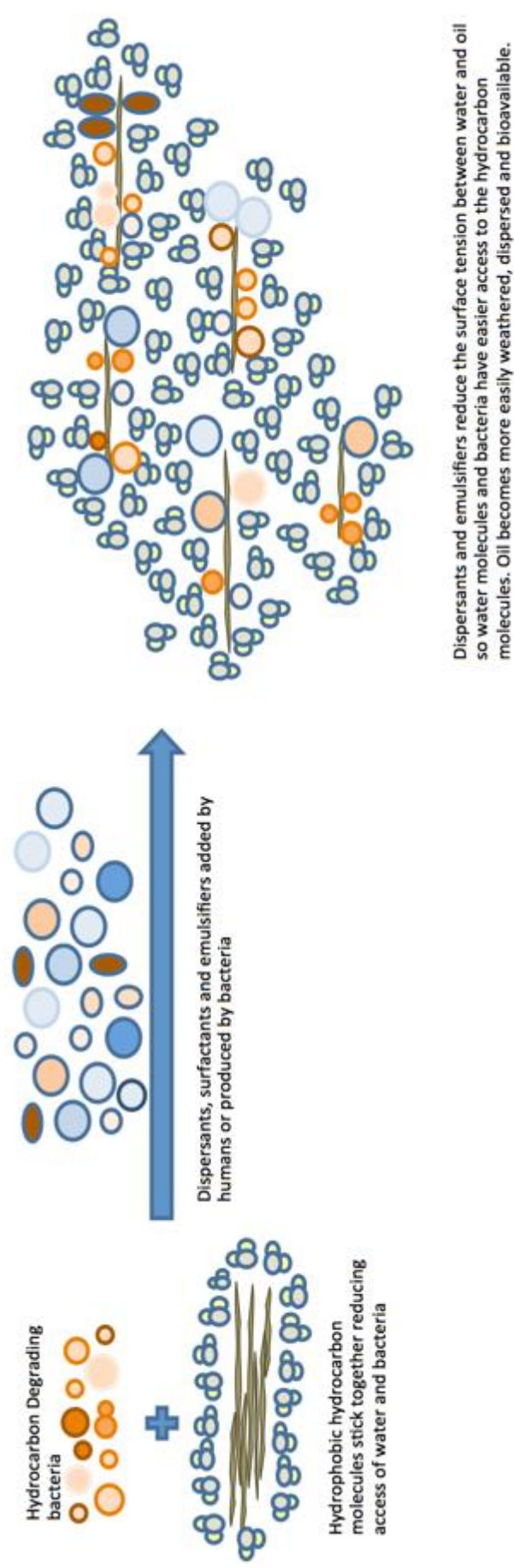
Unprecedented quantities, up to 7 million liters, of the dispersant Corexit EC9500A was applied by spraying on sea surface oil slicks and subsequently directly injected at the leaking wellhead near the seafloor during the Deepwater Horizon incident (National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011); this was after the dispersant Corexit 9527 was used initially. This surface and subsurface application of Corexit was reported to result in facilitated microbial biodegradation of the oil (Brakstad *et al.*, 2015) and the formation of large quantities of MOS and its subsequent sedimentation to the seafloor (Brooks *et al.*, 2015; Romero *et al.*, 2015; Daly *et al.*, 2016; Passow, 2016; Passow and Ziergovel, 2016). However, a study by Kleindienst *et al.* (2015a) in the Gulf of Mexico showed that certain members of the hydrocarbon-degrading community (specifically *Marinobacter*) could be inhibited by the application of Corexit. Whilst chemical dispersants, including Corexit, have been reported to trigger or enhance MOS formation (Baelum *et al.*, 2012; Fu *et al.*, 2014; Kleindienst *et al.*, 2015a; Passow, 2016; Chapter II of this thesis), the mechanisms underlying this process remain unclear. An intriguing observation in some of these studies has been the formation of flocs/aggregates when only dispersant, but no oil, was present (Kleindienst *et al.*, 2015a; Chapters II, III and IV of this thesis). These flocs/aggregates, which appear white to off-white in coloration, are quite ‘sticky’ or gelatinous when handled, and whilst they can be defined as a type of marine snow, they have received very little attention. In the event of an oil spill at sea, the surface or

subsurface spraying/injection of dispersants is likely to result in areas on the sea surface, or ‘pockets’ within the water column, where dispersant molecules would not directly interact with the oil. Free dispersant molecules would likely interact with DOC, as well as microbial cells, leading to the formation of marine dispersant snow (MDS). The composition, fate and impact of MDS in the marine environment remains largely unexplored.

Another interesting fact is the influence of dispersants on oil biodegradation. In a review article by Kleindienst *et al.* (2015b), the authors describe the differences that can be observed in biodegradation of two different types of crude oils (a Brent crude one-sweet and light crude oil and a light crude oil from Shell Refining Company) after amendment with a chemical dispersant. In the case of the Brent Crude oil, it was shown that the biodegradation was suppressed after the addition of the dispersant Corexit EC9500A comparing to when the dispersant was not added. However, in the case of the light oil from Shell, the biodegradation seemed stimulated by the addition of the same dispersant comparing to undispersed oil. In this thesis, in Chapter IV, it is possible to see that the nature of the dispersant has also an effect on the oil biodegradation.

Some studies showed the degree of toxicity of the dispersant Corexit 9500 used in the DWH on microzooplankton (e.g. Almeda *et al.*, 2014a), rotifers (e.g. Rico-Martinez, *et al.*, 2013), and coral larvae (e.g. Goodbody-Gringley *et al.*, 2013).

Some reports described its potential to be cytotoxic (Zheng *et al.*, 2014), including comparing its toxicity to other products such as the house cleaning products (Word *et al.*, 2015). For instance, dispersants were used as a response to the DWH to mitigate the surface oil slick, but they also contributed to the formation of deep-water oil plumes (Blackburn *et al.*, 2014). However, the potential effects of dispersants on the marine ecosystem are still poorly known. A better understanding and increase in research on the possible effects of dispersant in response to an oil spill is the potential light to learn how to apply them appropriately. That should ensure that the assessment and management of these dispersants is being more efficient and less harmful for the ecosystem. Dispersants together with oil and degrading microbial communities might be the perfect remedy as a response of an oil spill when applied under an appropriate protocol.



**Figure 1.8.** Hydrocarbons might not be always of easy access to Oil degrading bacteria. The addition of dispersants is supposed to reduce the surface tension between water and oil and potentially allow a better intake from the bacteria that may act as bioremediators.

### **1.7. Summary**

With the recent surge in oil and gas exploration into more challenging environments, the FSC is itself a forefront in this respect, an area of scientific relevance and of great interest for assessing the impacts of an oil spill to local ecosystems. By nature of its contrasting water masses and marked variations in physical-chemical hydrodynamic properties, the FSC offers an opportunity to empirically determine the response of its allochthonous microbial populations in the event a spill and use of chemical dispersants. It also provides the opportunity to draw parallels between the northeast Atlantic region and the Gulf of Mexico which, due to the DWH spill, had experienced a major anthropogenic contamination event. It has already been shown that the FSC surface waters develops an oil-degrading bacteria community as well as MOS formation with the amendment of oil and dispersant. Moreover, there is still debate on the effects of the dispersant on the marine ecosystem and its potential to form MOS and influence on oil-degrading bacterial communities. Archaea have been described to have an important role in oil degradation under anaerobic conditions (eg. Grossi *et al.*, 2000).

This thesis aims to address some of these questions, although, further studies in the fate of the oil and the response of the marine ecosystem to its presence would be crucial for developing a contingency plan in prospect of a potential oil spill occurring. The main objectives of this thesis are to study: 1) whether MOS would form in the FSC waters (Chapter II), 2) the role of EPS and nutrients in MOS formation (Chapter III), 3) the role of dispersants on MOS and MDS formation (Chapter II and III), 4) the effect of dispersants on the bacterial community composition and partially on oil biodegradation (Chapter IV). Other potential factors involved in MOS formation were also described although require further investigation (Annexe D).





## **Chapter II: Role of EPS, Dispersant and Nutrients on the Microbial Response and MOS Formation in the Subarctic Northeast Atlantic**

## **2.1. Overview**

In this study the formation of marine oil snow (MOS), its associated microbial community, the factors influencing its formation, and the microbial response to crude oil in surface waters of the Faroe-Shetland Channel (FSC) are reported for the first time. This work has been published in *Frontiers in Microbiology* (see List of Publications).

## **2.2. Hypothesis**

Although, MOS formation has only been described in few areas after major oil spills such as the Gulf of Mexico or the Baltic Sea, it is noted that it could occur worldwide in the event of an oil spill such as the marine snow. Its formation could be enhanced or minimised by environmental factors and the geographical area. Moreover, it is suggested that the addition of nutrients and/or dispersants will stimulate MOS formation.

## **2.3. Introduction**

With oil exploration expanding into more challenging environments, such as the Arctic and in deeper waters, it is necessary to instigate studies that aim to understand the fate of oil in these types of environments. As already mentioned in Chapter I, a region of interest is the Faroe-Shetland Channel (FSC) due to the fact that current oil extraction is increasing and expanding in this region. In the event of an oil spill in the FSC, the formation of MOS and its subsequent sedimentation to the seafloor by the process of MOSSFA could cause significant impacts to sensitive benthic ecosystems in these waters, such as rich communities of sponge fauna, the scleractinian coral *Lophelia pertusa*, polychaetes and anemones (Frederiksen *et al.*, 1992; Howell, 2010).

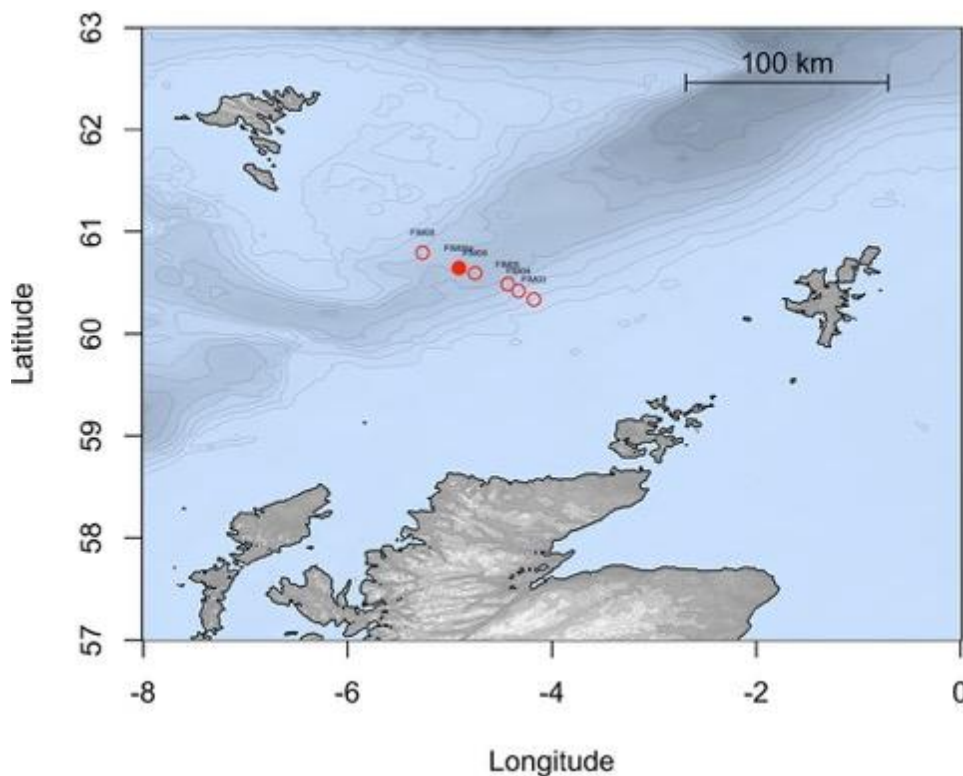
Since the formation of MOS and how oil-degrading communities respond to oil contamination can differ substantially both in space and time in the global ocean, this chapter investigates MOS formation and the microbial community response to crude oil in surface waters of the FSC, and compare and contrast this to the Gulf of Mexico. This is explored using a deep-sequencing approach with surface seawater from the FSC treated with and without nutrient and dispersant amendments, and discuss the role of natural seawater EPS, dispersants and nutrients in influencing MOS formation in the FSC, and of the MOS-associated bacterial community. The findings of this work are anticipated to provide a greater level of understanding on MOS formation and the microbial community response in the FSC as a reference of a contrasting Atlantic water body to the Gulf of

Mexico, and to help predict where the oil could end up on the seafloor in the event of an oil spill in this region.

## **2.4. Materials and Methods**

### **2.4.1. Field Samples**

During a research cruise on the MRV Scotia on 15 December 2015, sea surface water samples were collected from a depth of 5 m in the FSC ( $60^{\circ} 38.120' \text{ N}$ ,  $4^{\circ} 54.030' \text{ W}$ ; temp.  $8.7^{\circ}\text{C}$ ) at approximately 10 km from the Schiehallion oilfield. Sampling was conducted along the Fair Isle-Munken (FIM) line, which is a sampling transect that runs between the Faroe and Shetland Isles. The water samples fall within a water mass defined as Modified North Atlantic Water (MNAW) which originates from the Faroe Islands and travels in a south to south-westerly direction through the FSC before diverging at the south end of the FSC (Figure 2.1). The solid point (in red) shows the station sampled in this study from the offshore Schiehallion oilfield located approximately 175 km west of the Shetland Isles.



**Figure 2.1.** Map representing the stations studied along the FIM section of the Faroe Shetland Channel. The solid point shows the station sampled in this study.

MNAW is a warm and saltier Atlantic water mass compared to the underlying Arctic/Icelandic cold-water masses that are found at depths from 400 to 1500 m in the

FSC (Berx *et al.*, 2012). The water samples were immediately stored at 4°C aboard the ship in 10 L carboys and used within 1 week for the preparation of water-accommodated fractions (WAFs) and in enrichment experiments with crude oil, dispersant and/or nutrient amendment. Preliminary experiments done for this chapter can be found on Appendix B of this thesis.

#### **2.4.2. Water-accommodated Fractions**

A WAF is defined as a laboratory-prepared medium containing dispersed and solubilized crude oil hydrocarbons/droplets by mixing a bulk liquid (e.g., seawater) with crude oil, and subsequent removal of the non-dispersed/solubilised oil. Here, WAF was prepared following the method of Kleindienst *et al.* (2015b). Briefly, seawater collected from the FSC was first passaged through 0.22 µm filters to remove microbial cells, with the exception that the filtrate was not pasteurized as described in the method of Kleindienst *et al.* (2015b). This is because heat treatment could alter seawater chemistry, in particular the molecular integrity of DOM, such as bacterial EPS which was found to play a direct role in MOS formation during the DWH oil spill (Gutierrez *et al.*, 2013).

A 100-mL volume of the filter-sterilized seawater was amended with 17.6 mL of pre-filtered (0.22 µm) Schiehallion crude oil (provided by BP). Seawater amended with only dispersant comprised 100 mL of the filter-sterile seawater and 1.76 mL of Superdispersant-25, which is a UK-approved dispersant. The effective dilution of the dispersant in seawater (dispersant-to-oil ratio, v/v) was 1:10, which is a dilution that is recommended by the oil and gas industry (Approved oil spill treatment products, Government UK, July 2016). Chemically-enhanced WAF (CEWAF) medium was prepared with 100 mL of sterile seawater amended with 17.6 mL of filtered Schiehallion crude oil and 1.76 mL of Superdispersant-25 – the effective dilution of the dispersant in this treatment was also 1:10. These dilutions resulted in dispersant concentrations (~19 µg/L) that were comparable to concentrations observed in the DWH plume in situ (below detection to 12 µg/L) (Kujawinski *et al.*, 2011). Similarly, the concentration of TPH in the deepwater plume ranged from 2 to ~440 µg/L (Wade *et al.*, 2013) and in the water-accommodated fraction (WAF) and CEWAF microcosms, the TPH concentration were in a similar range, from 30 to 300 µg/L. The various mixtures of sterile seawater (SW) amended with oil (WAF), oil+dispersant (CEWAF) and solely dispersant (SW+D) were mixed on a rotary magnetic stirrer at 140 rpm for 48 h at 7°C in the dark in clean sterile (acid-washed) 500-mL glass bottles. The mixtures were allowed to stand for 1 h and then

the aqueous phases (avoiding non-dispersed/solubilized oil or dispersant) were sub-sampled into clean (autoclaved and acid washed with 5% nitric acid) screw-capped glass tubes with Teflon caps. These WAF, CEWAF and SW+D solutions were stored at 4°C and used within 48 h for the various microcosm experiments. Treatments containing nutrients – i.e., seawater+nutrients (SW+N) and CEWAF+nutrients (CEWAF+N) – were amended with 10 µM ammonium chloride, 10 µM sodium nitrate and 1 µM potassium phosphate (final concentrations).

### 2.4.3. Microcosm Setup and Sampling

To examine the microbial response and formation of MOS in sea surface waters of the FSC when exposed to crude oil, dispersant and/or nutrients, a roller-bottle design was used as previously described (Gutierrez *et al.*, 2013).

**Table 2.1. Treatments used during the experiment.**

<b>Treatment</b>	<b>Description</b>
<b>SW</b>	Sea water
<b>SW+N</b>	Sea water+ nutrients
<b>SW+Disp</b>	Sea water+ dispersant
<b>WAF</b>	Sea water+ Oil
<b>CEWAF</b>	Sea water+ Oil+ dispersant
<b>CEWAF+N</b>	Sea water+ Oil+ dispersant+ nutrients

For this, Pyrex© glass bottles (38 × 265 mm) with the different treatments were maintained in constant and gentle motion in order to simulate pelagic seawater conditions (Jackson, 1994). Each treatment was run in duplicate and comprised of 42.75 ml of filter-sterile WAF, dispersant-only, or CEWAF (with/without nutrients) added to 150 ml of unfiltered natural seawater collected from the FSC. In addition, two oil-dispersant untreated controls were setup and run in parallel: one comprised seawater alone with no other additions (SW), and the second of seawater with only nutrients added (SW+N). Treatments and controls were each established in duplicates and incubated at 7°C (approx. sea surface temp. in the FSC at the time of sampling) and in the dark at a rotation speed of 15 rpm. The treatments and controls were sampled at five time points over the course of 6 weeks: T<sub>0</sub> at day 0, T<sub>1</sub> after 1 week, T<sub>2</sub> after 2.5 weeks, T<sub>4</sub> after 4 weeks, and T<sub>6</sub> after 6 weeks. At each sampling time, the bottles were placed in an upright position to capture a photographic record of MOS formation. Sub-samples of water were also

withdrawn for DNA extraction and DAPI (4',6-diamidino-2-phenylindole) cell counts (see below); care was taken not to capture MOS particles in order to quantify bacterial abundance in the free-living fraction. Visible aggregates were carefully withdrawn using glass Pasteur pipettes and transferred to 1.5-ml microcentrifuge tubes for staining with the cationic copper phthalocyanine dye alcian blue (AB) at pH 2.5 (Alldredge *et al.*, 1993) or the amino acid-specific dye Coomassie Brilliant Blue G (CBBG) at pH 7.4 (Long and Azam, 1996). AB is used for staining acidic sugars of EPS or transparent exopolymer particles (TEP) in seawater, whereas CBBG is used for staining the proteinaceous component of these polymeric substances. Following staining, the aggregates were washed by transferring them through several droplets of sterile water prior to their examination under the light microscope. To directly examine the prokaryotic community under the microscope, MOS particles were also stained with acridine orange (AO) (Francisc *et al.*, 1973) for imaging with a FITC filter on a Zeiss Axioscope epifluorescence microscopy (Carl Zeiss, Germany). Only the light intensity and the contrast of the pictures taken under the microscope were modified with Image J software (Rueden *et al.*, 2017). Moreover, the treatments and controls were observed daily over the course of the experiment to detect any visual change, such as turbidity, emulsion and/or MOS formation. This step was only made for the observation of potential physical changes in the tubes.

#### **2.4.4. Genomic DNA Extraction**

DNA was extracted from the original natural seawater collected from the FSC and from subsamples taken from each of the treatments and controls of the 6-week roller-bottle experiment. For this, ten milliliter samples were filtered using a glass column filtration system (Millipore) with 45 mm polycarbonate membrane filters (0.22  $\mu$ m pore size; Isopore) and the filters stored at  $-20^{\circ}\text{C}$ . The membrane filters were cut into three equal parts, and then each part placed into 1.5-mL microcentrifuge tubes and ground up with liquid nitrogen. The liquid nitrogen was allowed to completely evaporate from each tube and the contents extracted according to the method of Tillett and Neilan (2000). Purified DNA was stored at  $-20^{\circ}\text{C}$  for subsequent molecular analysis.

#### **2.4.5. Barcoded Amplicon Metagenomic Sequencing and Analysis**

Barcoded 16S rRNA gene MiSeq sequencing, targeting the V3-V4 hypervariable region, was employed to analyze the bacterial community over the 6-weeks duration of the experiments at time points  $T_0$ ,  $T_2$ ,  $T_4$  and  $T_6$ . The 16S rRNA gene was amplified in

duplicate 50 µl reactions. Each reaction comprised 32 µl molecular biology grade water, 10 µl 5x MyTaq polymerase reaction buffer, 2.5 µl 4 µM primer mix, 0.5 µl MyTaq Enzyme (2.5U; BioLine), 3 µl DMSO (6%), and 2 µl gDNA. The primers used were 341f-CCTACGGGNGGCWGCAG and 785r-GGACTACHVGGGTWTCTAAT. Both primers also had Illumina MiSeq overhangs attached to their 50 ends. Barcodes were not added at this point of PCR. Thermocycler conditions for this first round of PCR were an initial denaturation of 96°C for 1 min, 32 cycles of 96°C for 15 sec, 55°C for 15 sec, and 72°C for 30s, and a final extension at 72°C for 3 min. PCR clean-up was performed using 20 µl of the PCR product, and adding 1 µl FastAP (1U), 0.5 µl Exonuclease I (10U; both Thermofisher) and 3.5 µl molecular grade water. Conditions for the PCR clean-up reaction were 45 min at 37°C followed by 15 min enzyme denaturation step at 85°C. The purified PCR amplicons were then subjected to a second-step PCR at the sequencing facility for the addition of the Golay barcodes, which were unique to each treatment. All samples were sequenced via the Illumina MiSeq platform (Illumina 2 × 250 V.2 kit) at the University of Liverpool Centre for Genomic Research<sup>1</sup>; sequences were demultiplexed prior to receipt at our laboratory. Subsequent processing was conducted using the MiSeq SOP (accessed: September 2016) cited within the MOTHUR program (Kozich *et al.*, 2013). In brief, single end reads were examined using MOTHUR (v1.36.1).

Contiguous sequences were constructed from paired end sample reads. All sequences with any ambiguities or homopolymers longer than eight bases were excluded from further analysis. All remaining sequences were aligned against a SILVA compatible database. All sequences were trimmed to a maximum length of 465 bases before chimeric sequences were identified and removed using UCHIME (Edgar *et al.*, 2011). The taxonomic identity of sequences was determined by comparison to a MOTHUR formatted RDP database (v.14). Any sequence returned as unknown, chloroplast or mitochondrial were removed from further downstream analysis. Operational taxonomic units (OTUs) were clustered based on 97% sequence identity and subsampled to 35,000 sequences per sample to eliminate sampling bias during subsequent diversity examination. All sequences were deposited in SRA repository under accession number SAMN06246901.

#### **2.4.6. Prokaryotic Cell Counts**

To quantify prokaryotic (bacteria and archaea) cell counts, DAPI (4', 6-diamidino-2-phenylindole) was used as a staining technique. For this, sub-samples of water from each treatment and control of the roller-bottle incubations were fixed with

3.7% formaldehyde and stored at 4°C for a maximum of 2 weeks. The collection of MOS particles, as part of these water samples, was avoided here, as the accurate enumeration of cells associated with MOS was not feasible due to oil auto-fluorescence and obscured visualization of cells due to the agglomerate matrix. For each fixed water sample, 5 ml was filtered (0.22 µm) onto gridded (3 mm × 3 mm) polycarbonate filters – this volume was adjusted in order to achieve 10–150 cells per grid. The filters were mounted onto glass slides and the cells stained with DAPI (1 µg/ml) for 20 min and then counted under the Zeiss Axioscope epifluorescence microscope (Carl Zeiss, Germany). A minimum of 10 grids were randomly selected and photographed for counting of cells. The number of cells counted was calculated using the equation (1) below described in (Wetzel and Likens, 1991):

$$N = \left( \frac{nb}{nSq} \right) Vf \left( \frac{A}{ASQ} \right) \quad (1)$$

where:

$N$  is the total number of bacteria per mL

$nb$  is the number of bacteria counted

$nSq$  is the number of squares counted

$Vf$  is the volume of seawater filtered

$A$  is the effective filter area

$ASq$  is the area of one square of the grid

#### 2.4.7. Statistical Analyses

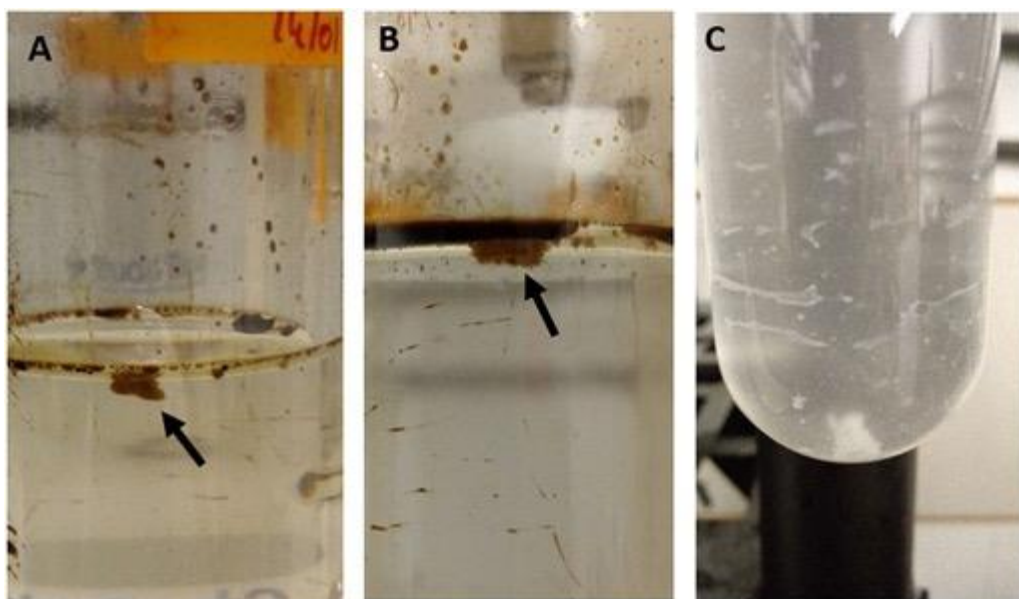
Relative abundances of sequences obtained using MiSeq were compared using an NMDS plot to visualize β-diversities of each sample for both treatment and time point. An ANOSIM analysis was conducted to determine if there was any significant difference between treatments employed. Further examination of the α-diversity was achieved by generation of rarefaction curves, based on 97% sequence similarity. Moreover, Shannon-Weiner diversity indices ( $H'$ ) were generated using base 10 and compared using an analysis of variance to determine significant differences between diversity of treatments and time points sampled. All data was log transformed to meet the assumptions of parametric analysis.



## **2.5. Results**

### **2.5.1. MOS Formation**

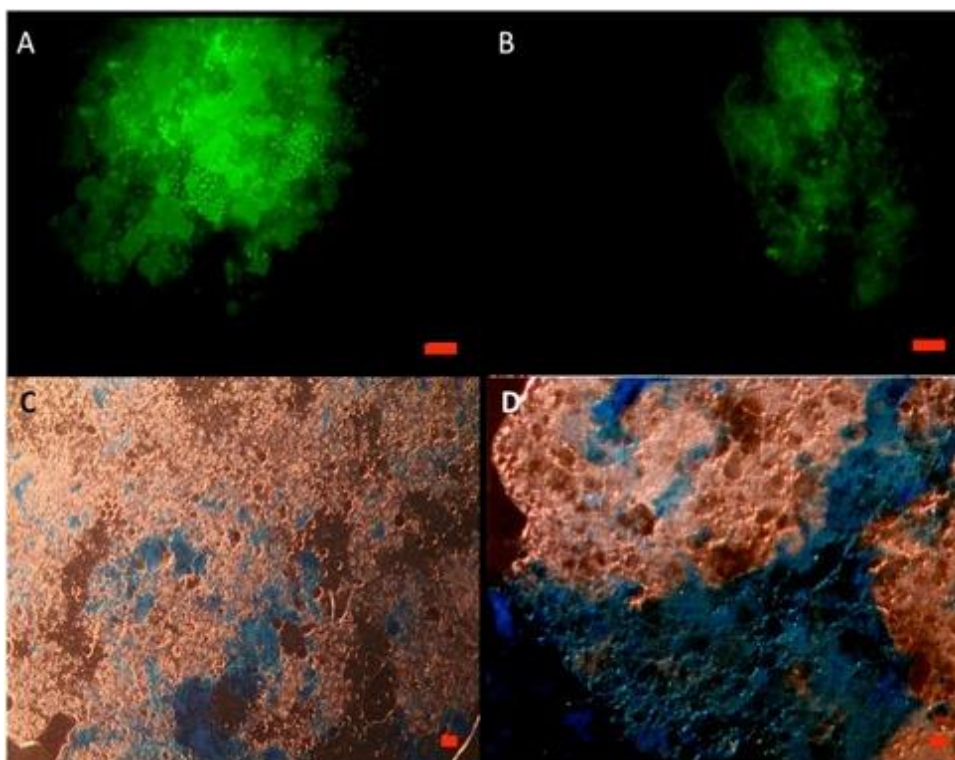
In the roller-bottle microcosm incubations, a rapid formation of MOS was observed within 5 days in the CEWAF+N treatment, and within 7 days in the CEWAF treatment. In both treatments the MOS particles appeared brownish, round and of ‘fluffy’ texture (Figures 2.2 A and B). Initially, the aggregates were small (<3 mm in diameter) and exhibiting amorphous definition. With the naked eye, small oil droplets could be seen associated within the amorphous matrix of the MOS aggregates from these treatments. Over the course of these roller-bottle incubations, the aggregates were observed to become progressively less buoyant, and by week 6 they settled to the bottom of the glass tubes when held in an upright position. The size of the MOS aggregates in these incubations (CEWAF and CEWAF+N) also increased over time (from initially 2–3 mm to ~2 cm after 4 weeks) and it is posited that smaller aggregates had merged together since it was observed that the absolute abundance of MOS particles (i.e., that could be counted by visual observation) had decreased over time. By week 4, aggregate size appeared to stabilize (1–2 cm average aggregate size) and remained unchanged thereafter.



**Figure 2.2.** Marine oil snow aggregates shown floating at the surface of the CEWAF (A) and CEWAF+N (B) roller-bottle incubations, and Marine Dispersant Snow (MDS) aggregates shown settled at the bottom of the bottles in the SW+D treatments (C).

In the treatment of seawater with only dispersant (SW+D), however, the formation of large (size range 0.5–3 cm) white aggregates occurred within 3 days (Figure 1.2C), for

which I propose to define hereafter as ‘marine dispersant snow’ (MDS). Aggregates of MDS were approximately 2–3 times larger in size compared to MOS aggregates that formed in the CEWAF and CEWAF+N treatments. As similarly observed for MOS particles in these treatments, MDS aggregates progressively lost their buoyancy and eventually, by week 2, settled to the bottom of the glass tubes when held in an upright position (Figure 2.2C). Manipulation of selected MDS aggregates on a microscope slide revealed they exhibited quite viscous/gelatinous characteristics. In contrast, the formation of MOS was not observed in the WAF treatment, and no marine snow particles formed in the SW control incubations. However, in the SW+N treatment the formation of marine snow (no oil) was observed and these particles were comparatively small (1–2 mm) and remained so for the duration of these experiments.

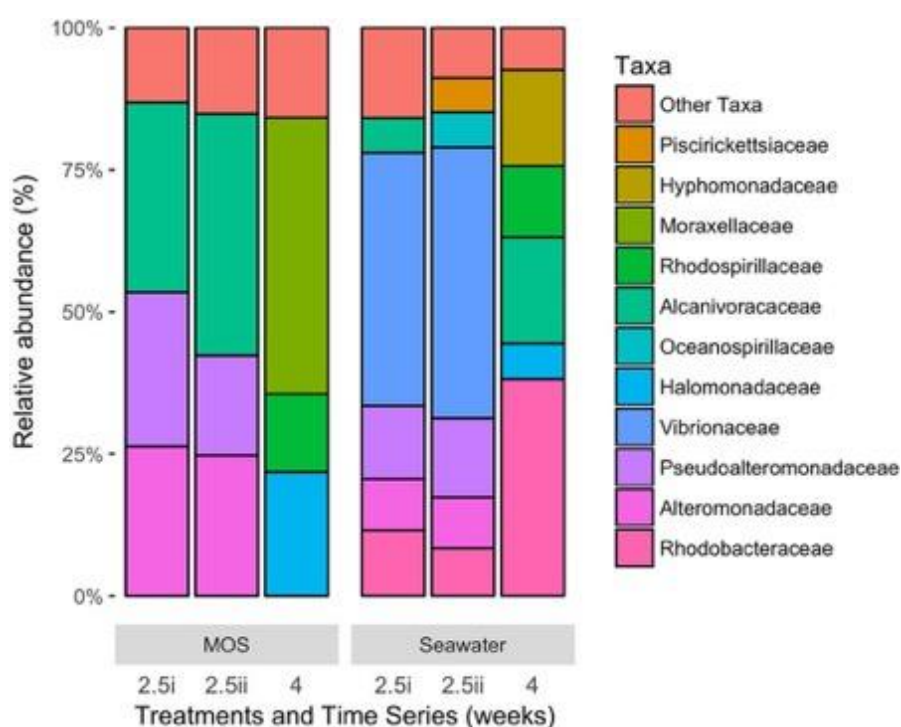


**Figure 2.3.** Formation of MOS and marine snow in the roller bottle incubations. Under epifluorescence microscope after staining with acridine orange, MOS (A) which formed in the CEWAF ( $\pm$ nutrients) treatments was populated with associated prokaryotic cells (small green dots) and oil droplets (large green spherical/irregular blobs). Marine snow (B) that formed in the SW+N treatments contained few associated prokaryotic cells. Under the light microscope, MOS stained with Coomassie Brilliant Blue G (C) and Alcian Blue (D) showing the presence of EPS. Bar, 10  $\mu$ m.

They were also observed to be extremely fragile and disintegrate when the incubation chamber was gently shaken. When viewed under the epifluorescence microscope with AO staining, MOS aggregates from the CEWAF and CEWAF+N treatments appeared as amorphous ‘fluffy’ particles with associated oil droplets (large green blobs; average size range 5 to >20  $\mu\text{m}$  diameter) and represented foci for the attachment of prokaryotic cells (Figure 2.3A). Similarly, MDS aggregates also showed the presence of associated prokaryotic cells (results not shown). Marine snow particles (without oil) that formed in the SW+N treatment, however, were observed to contain markedly fewer prokaryotic cells (Figure 2.3B). When viewed under the light microscope with the aid of dark field illumination, MOS aggregates partially stained with CBBG (Figure 2.3C) and AB (Figure 2.3D).

### 2.5.2. Bacterial Community Composition of MOS

Barcoded 16S rRNA Illumina MiSeq technology was used to analyze the bacterial community associated with MOS and of that in the surrounding (not associated with MOS) seawater.



**Figure 2.4.** Bacterial community composition at family-level classification of MOS compared to that in the surrounding seawater in the CEWAF+N treatment at weeks 2.5 and 4.

This procedure was limited to only the CEWAF+N treatment because MOS aggregates that formed in this treatment maintained their structural integrity and did not disintegrate when handled; MOS aggregates from the other treatments were found to be quite fragile and handling them during the initial processing steps for MiSeq sequencing resulted in them breaking up and completely disintegrating.

Figure 2.4 shows the bacterial community structure – at family-level classification – of MOS formed in the CEWAF+N treatment at weeks 2.5–4, presented here alongside the community of the surrounding seawater from the same roller-bottle incubation. Of a total of up to 448,754 high quality partial 16S rRNA gene sequences, the MOS bacterial community at the 2.5-week time point showed a clear dominance of members within the *Alcanivoracaceae*, *Alteromonadaceae* and *Pseudoalteromonadaceae* – respectively, on average 38.0, 25.5 and 22.4% of the total MOS-associated community. Minor representation (of >1%) included phyla within the *Rhodobacteraceae* (2.9%), *Rhodospirillaceae* (2.3%), *Vibrionaceae* (2.1%) and *Piscirickettsiaceae* (1.2%). In contrast, the bacterial community of the seawater surrounding MOS aggregates from this same CEWAF+N treatment at week 2.5 was dominated by phyla within the *Vibrionaceae* (46.1%), with high contributions also by high contributions also by *Pseudoalteromonadaceae* (13.4%), *Rhodobacteraceae* (10.0%), *Alteromonadaceae* (9.0%), *Oceanospirillaceae* (5.6%), *Piscirickettsiaceae* (5.5%) and *Alcanivoracaceae* (5.3%).

An analysis of the major groups at the level of genus revealed some interesting groups that dominated the community associated with MOS in the CEWAF+N treatment when analyzed at the T<sub>2</sub> and T<sub>4</sub> time points compared to that in the surrounding seawater. At T<sub>2</sub>, MOS was dominated by members of the genera *Alcanivorax* (33–42%), *Pseudoalteromonas* (17–27% of total sequence reads), *Alteromonas* (25%), with minor representation by *Sulfitobacter*, *Vibrio*, *Thalassospira*, *Cycloclasticus* and *Mesonina* (collectively contributing <10% of total reads). At the T<sub>4</sub> time point, MOS was dominated by members of the genera *Psychrobacter* (48.4% of total sequence reads), *Cobetia* (21.6%), *Thalassospira* (13.8%), with minor representation by *Pseudoalteromonas* (4.4%), *Alcanivorax* (2.7%), *Cycloclasticus* (1.6%) and *Marinobacter* (1.2%).

In terms of the number of 16S rRNA reads that were found enriched on MOS compared to their abundance in the surrounding sea water at the T<sub>4</sub> time point, *Psychrobacter* was 970-fold higher in abundance, *Marinobacter* 20-fold higher, *Halomonas* 8.5-fold higher, *Pseudoalteromonas* 7.5 fold-higher, *Cobetia*, *Cycloclasticus* and *Vibrio* 3.5-fold higher, *Alteromonas* 3-fold and *Thalassolituus* 1.5-fold higher.

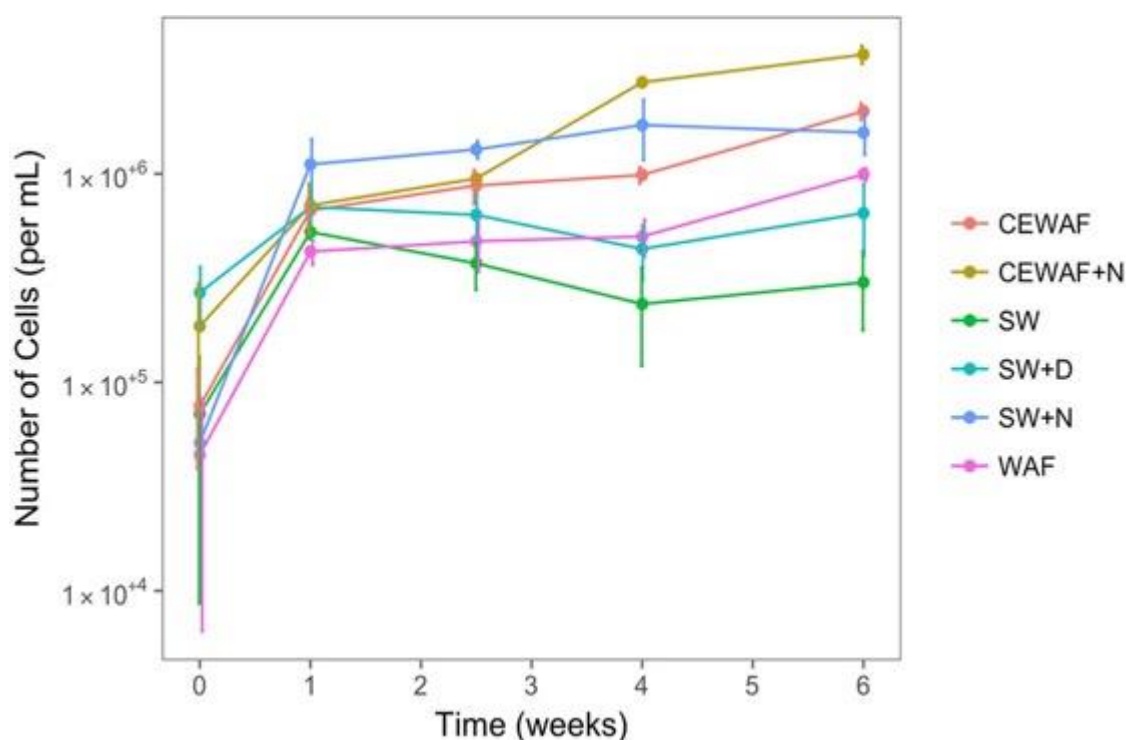
### 2.5.3. Bacterial Community Dynamics in the Various Treatments

To assess the free-living (not associated with MOS) prokaryotic community dynamics in the different treatments amended with and without nutrients, dispersant or crude oil, DAPI counts were determined over the 6-week duration of these experiments at time-points T<sub>0</sub> (start day of the experiment), T<sub>1</sub> (after 1 week), T<sub>2</sub> (after 2.5 weeks), T<sub>4</sub> (after 4 weeks) and T<sub>6</sub> (after 6 weeks). As shown in Figure 2.5, prokaryotic cell abundance across all six treatments at the start of the experiment (T<sub>0</sub>) was  $0.8\text{--}15.0 \times 10^4$  cells/mL, and as expected cell abundance in the untreated control (SW) remained low relative to the other treatments throughout the 6-week duration of these experiments. Similarly, low prokaryotic cell abundances were achieved in the SW+D and WAF treatments ( $6.9 \times 10^5$  and  $9.9 \times 10^5$  cells/mL, respectively). In the SW+N treatment, however, cell numbers showed the highest increase within the 1st week, and then slowing down to a steady increase over the proceeding 3 weeks, and reaching maximal abundances by week 4 ( $1.7 \times 10^6$  cells/mL). Prokaryotic cell abundances in the CEWAF and CEWAF+N treatments followed a similar increasing trend initially, and their dynamics diverged after about 2 weeks. Cell abundances in the CEWAF+N treatment showed the most notable increase compared to the other treatments, reaching  $3.7 \times 10^6$  cells/mL by week 6.

Although DAPI counts demonstrated an expected pattern for prokaryotic dynamics in these treatments, the counts are likely to be somewhat underestimated with particularly the treatments where MOS had formed due to the high numbers of DAPI-stained prokaryotic cells associated with MOS particles (Figures 2.3 A and 2.3B). The enumeration of the cells was practically impossible to count accurately because of their density and localization within and on the surface of the MOS aggregates. The diversity of the bacterial communities in the surface seawater of the FSC and their response to and dynamics in the various treatments (SW, SW+N, SW+D, WAF, CEWAF, CEWAF+N)

was assessed using Illumina MiSeq technology and shown at family-level classification in Figure 2.6.

At the commencement of these experiments (denoted by time point  $T_0$ ), the community was initially dominated by groups within the *Alteromonadales* and *Rhodobacterales* – collectively 96% of total sequence reads. The major genera of this  $T_0$  community constituted *Colwellia* (33.7%), *Sulfitobacter* (28.2%), *Pseudoalteromonas* (10.5%), *Alteromonas* (2.7%) and other members of the family *Alteromonadaceae* (22.4%). Other phyla, such as the hydrocarbonoclastic bacteria *Alcanivorax*, *Cycloclasticus*, *Marinobacter* and *Thalassolituus*, as well as *Halomonas* that, like *Alteromonas* and *Pseudoalteromonas*, are recognized producers of EPS were also present though in low abundance ( $\leq 0.5\%$  for each).



**Figure 2.5.** Average of prokaryotic (bacterial and archaeal) cell numbers from roller-bottle incubations of the different treatments with sea surface water from the FSC amended with or without nutrients, dispersant and/or crude oil (as WAF) and their respective standard deviation bars. SW, seawater; SW+N, seawater with nutrients; SW+D, seawater with dispersant; WAF, water accommodated fraction; CEWAF, chemically enhanced WAF; CEWAF+N, chemically enhanced WAF.

This bacterial community of the FSC sea surface in the untreated control (SW) maintained a relatively consistent structure throughout the 6-week duration of these experiments. Rarefaction analysis of a sub-set (35,000 sequences) of the 16S rRNA gene sequences showed that for no treatment was saturation of sequencing reached (Figure 2.7). The OTU richness of each treatment ranged between 520 and 1,010 of identified OTUs at T<sub>1</sub>, and upon the termination of the experiment (T<sub>6</sub>) all the treatments exhibited, with the exception of SW+N and CEWAF+N, a reduction in the number of OTUs with nutrients.

Overall the  $\alpha$ -diversity indices (Shannon-Weiner H') for each treatment indicated that only SW+D and SW+N had higher diversities than were measured for the SW controls (Figure 2.8A; ANOVA,  $F_5 = 0.05326$ ,  $p < 0.01$ ). Moreover, diversity also declined overall during the period of the experiment (Figure 2.8B; ANOVA,  $F_1 = 0.1192$ ,  $p < 0.01$ ). The similarity between treatments and samples therein can be visualized in Figure 2.9. This indicated the similarity of each sample to all other samples examined and confirmed that there was significant dissimilarity between the bacterial communities within the treatments (ANOSIM,  $R = 0.6624$ ,  $p < 0.001$ ). Here it can be observed that the  $\beta$ -diversity most prominently differs between water types and not time points measured. Most distinct are the SW+N and SW+D treatments.

Within the first week, this complex community of the FSC surface seawater became overlayed by opportunistic bacteria that were stimulated by the presence of either nutrients, dispersant and/or crude oil. The community in the SW+N control treatment showed a rapid enrichment of members within the order *Alteromonadales*, mainly of the genus *Alteromonas*, which remained as a dominant group (21–60%) until the termination of the experiment when their abundance decreased to ca. 8% at week 6. To a lesser extent, *Neptuniibacter* within the order *Oceanospirillales* and members of the family *Flavobacteriaceae* were also dominant groups that bloomed by week 2 and collectively persisted as the most dominant groups for the remaining duration of these incubations. Furthermore, a progressive enrichment of members within the *Rhodobacteraceae* occurred by week 1 and reached 13% of the total community by week 6. Similarly in the SW+D treatment, members of the *Alteromonadales* dominated and persisted for the remaining duration of the experiment. However, the community showed clear, though lower, representation of groups within the *Rhodospirillales*, mainly

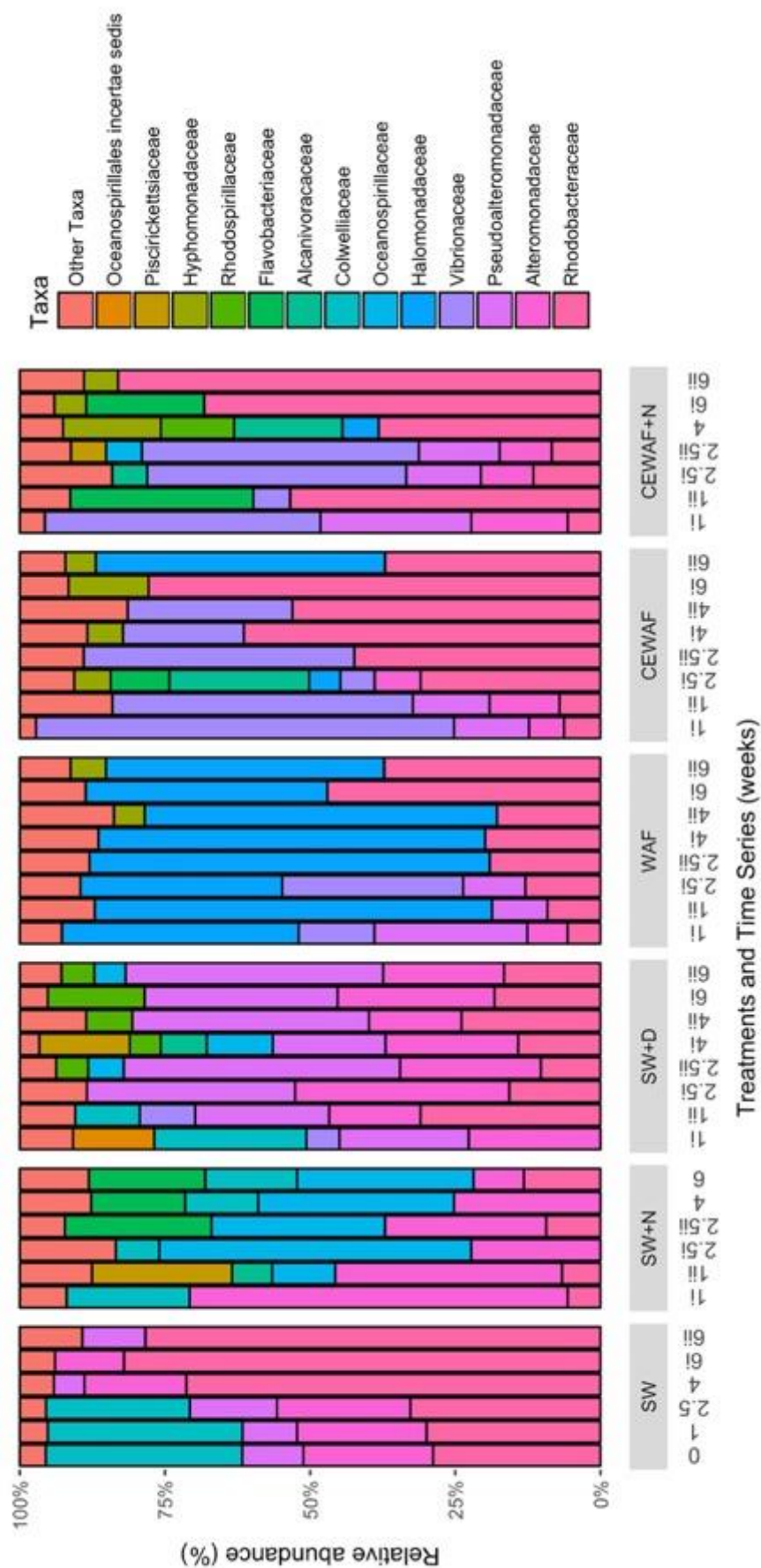


contributed by the genus *Thalassospira*, and by members of the *Rhodobacterales* and *Oceanospirillales*, and by phyla of the class *Gammaproteobacteria* that included the genus *Vibrio*, although it bloomed (up to 10%) in only the first week. Compared to the SW and SW+N controls, the presence of dispersant in the SW+D treatment caused a clear reduction (to <1%) in the abundance of *Colwellia* by week 2.

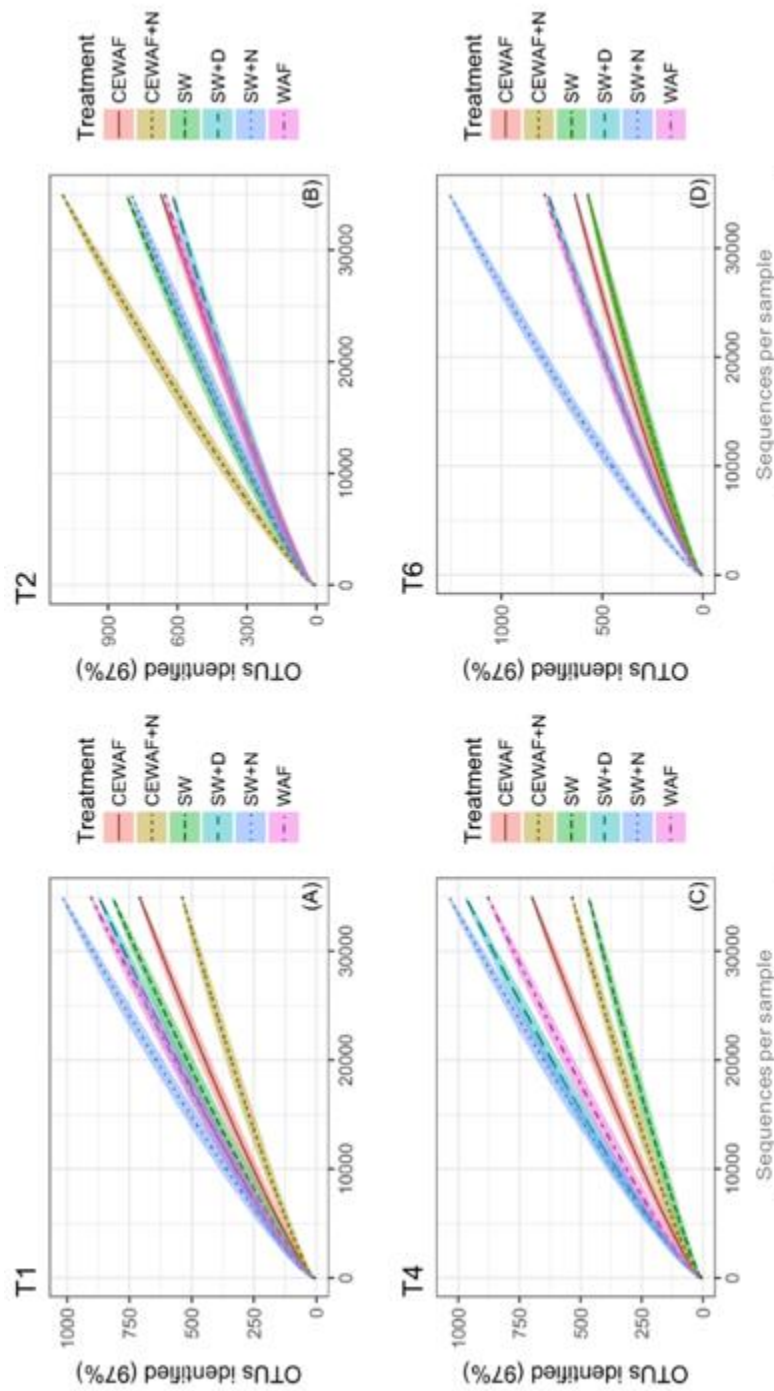
In the WAF treatment, where the FSC seawater was amended with crude oil in the form of a WAF, the microbial response was distinctly different compared to the SW, SW+N and SW+D treatment. Within 1 week the community of the WAF treatment became strongly dominated by members of the *Oceanospirillales*, largely of the genus *Halomonas* (34–65%) that persisted as the major group for the remaining duration of the experiment. Other major groups included members of the *Alteromonadales*, largely of the genus *Pseudoalteromonas* but that bloomed in week 1 (up to 25%) and progressively decreased in abundance thereafter. Members of the family *Rhodobacteraceae* increased in abundance from week 1, reaching maximal levels (42.2%) by week 6, and a short-lived bloom of *Vibrio* occurred within weeks 1–2. As observed in the SW+D treatment, the abundance of *Colwellia* dramatically decreased in abundance, this time within week 1, in the WAF treatment. The bacterial community response in the CEWAF and CEWAF+N treatments contrasted substantially to the controls and other treatments. Here, the bacterial community response to the oil and dispersant (in the presence or absence of nutrients) revealed a more complex pattern of microbial succession, especially when viewed at the genus-level classification, over the 6-week duration of these incubations.

Within the first week, both treatments showed a strong bloom for members of the *Vibrionales*, principally the genus *Vibrio* that then decreased in abundance by week 3 in the CEWAF+N treatment, and by week 4 in the CEWAF treatment. In parallel, a progressive decrease in the abundance for members of the order *Alteromonadales* occurred within the first few weeks, principally contributed by the genera *Colwellia* community, though this time contributed by the genera *Pseudoalteromonas* and *Alteromonas*, and their dominance. As similarly observed in the SW+D and WAF treatments, the abundance of *Colwellia* in these CEWAF and CEWAF+N treatments dramatically decreased within week 1 and remained in very low abundance (<0.4%) for the remaining duration of these experiments.

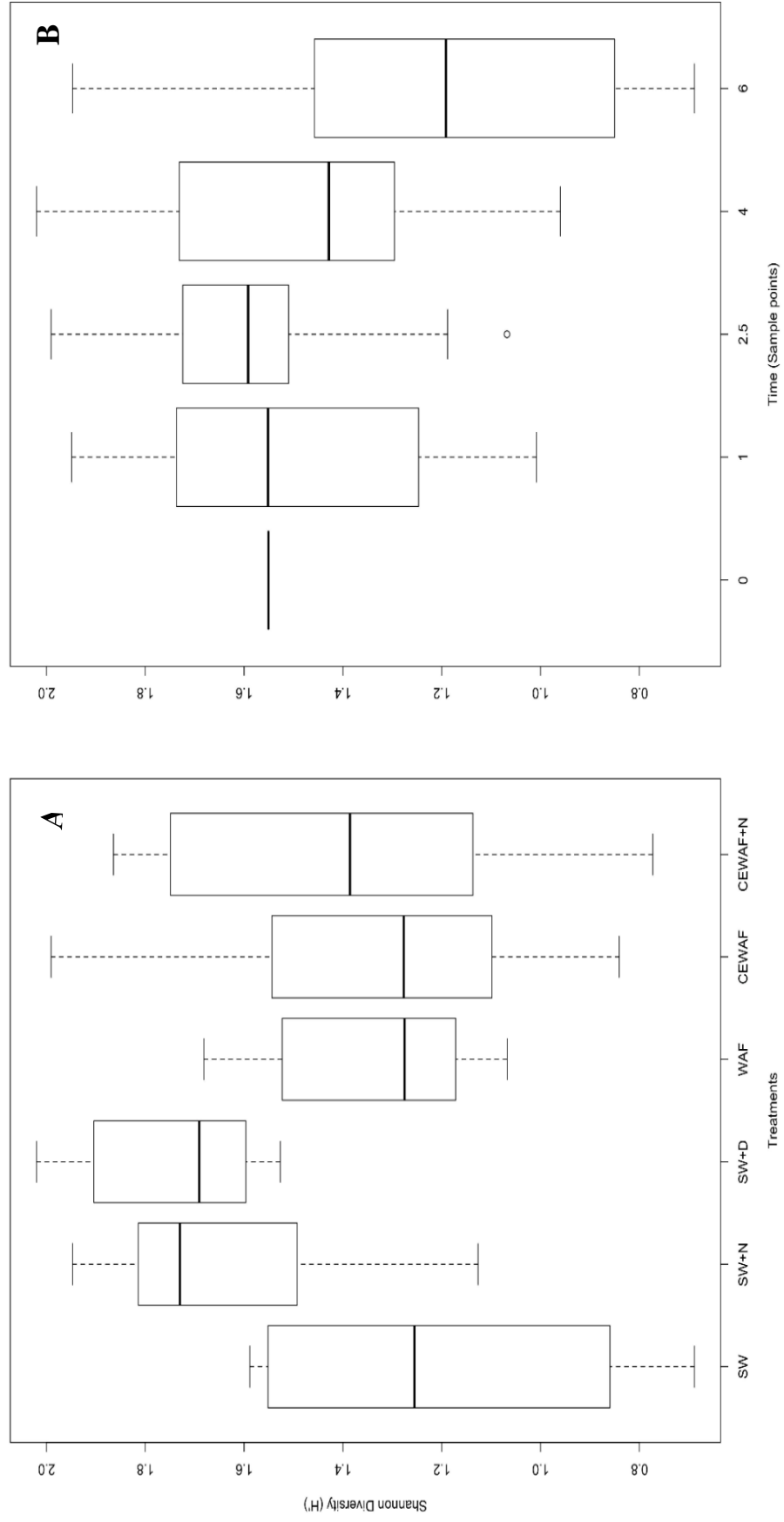




**Figure 2.6.** Bacterial community composition at family level classification of each of the different treatments over the 6 week incubation period that the roller-table experiments were run. SW, seawater; SW+N, seawater with nutrients; SW+D, seawater with dispersant; WAF, water-accommodated fraction; CEWAF, chemically enhanced WAF; CEWAF+N, chemically enhanced WAF with nutrients.

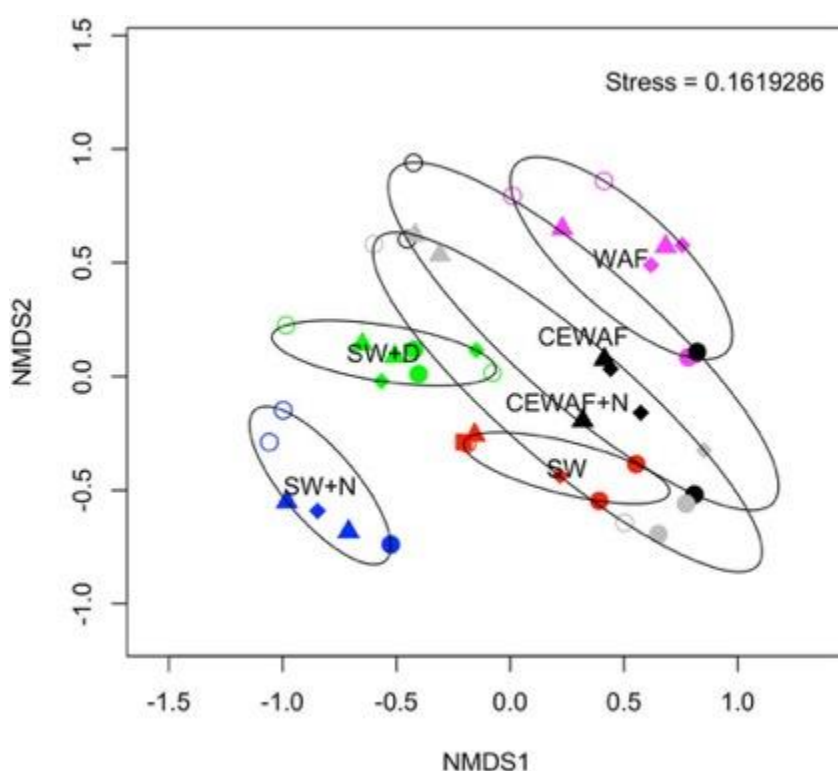


**Figure 2.7.** Rarefaction curves signify the  $\alpha$ -diversity of all treatments across all sampling times. OTUs are identified based on similarity of the 16S rRNA gene (97%). SW, seawater; SW+N, seawater with nutrients; SW+D, seawater with dispersant; WAF, water-accommodated fraction; CEWAF, chemically enhanced WAF; CEWAF+N, chemically enhanced WAF with nutrients. Sample points are: T<sub>1</sub>, (A); T<sub>2</sub>, (B); T<sub>4</sub>, (C); T<sub>6</sub>, (D). SW, seawater; SW+N, seawater with nutrients; SW+D, seawater with dispersant; WAF, water-accommodated fraction; CEWAF, chemically enhanced WAF; CEWAF+N, chemically enhanced WAF with nutrients. Sample points are: T<sub>1</sub>, (A); T<sub>2</sub>, (B); T<sub>4</sub>, (C); T<sub>6</sub>, (D).



**Figure 2.8.** The Shannon-Weiner diversity indices are presented here as a boxplot comparing the  $\alpha$ -diversity between water treatments (**A**) and time points (**B**) measured in this study.

On the other hand, *Pseudoalteromonas*, also a member of the order *Alteromonadales*, remained at relatively high abundance for the first week in the CEWAF treatment (13.2%) and second week in the CEWAF+N treatment (13.4–14.1%) before decreasing thereafter, whereas *Marinobacter* bloomed intermittently at weeks 1–2 in, respectively, the CEWAF (6.5%) and CEWAF+N (6.2%) treatments. Short-lived blooms of *Mesonina*, of the class *Bacteroidetes*, occurred at week 2 and in weeks 4–6 in, respectively, the CEWAF and CEWAF+N treatments. Notably, the enrichment of the obligate alkane-degrader *Alcanivorax* occurred in the CEWAF treatment during the first 2 weeks and which was prolonged into week 4 in the CEWAF+N treatment before the abundance of these organisms dissipated thereafter in both of these treatments to background levels.



**Figure 2.9.** Non-metric multidimensional scaling (NMDS) plot showing the similarity of each sample. The stress achieved is indicate in the top right of the plot. Symbol colours signify the original treatment: SW (red); SW+N (blue); SW+D (green); WAF (purple); CEWAF (black); CEWAF+N (grey). Each time point is represented by a different symbol: T<sub>0</sub> (square); T<sub>1</sub> (open circle); T<sub>2</sub> (triangle); T<sub>4</sub> (diamond); T<sub>6</sub> (closed circle). Ellipses shown surrounding symbols represent grouping the treatments types with 95% confidence interval.

Members of another alkane-degrader, *Thalassolituus*, and of the PAH-degrader *Cycloclasticus* were also enriched in only the CEWAF+N treatment, occurring during week 2. A less pronounced enrichment of members within the genus *Thalassospira*, for which some species have been described to degrade hydrocarbons, occurred in both these treatments and only toward the end of these incubations.

## **2.6. Discussion**

To my knowledge this is the first study examining the formation of MOS in northeast Atlantic waters. The study is specifically focused on the FSC where subsurface oil extraction is currently occurring and where exploration for oil in deeper waters (>1000 m depth) within this channel may expand in the near future. This work is important given that an oil spill in the deep waters of the FSC could produce a similar oil spill as occurred during the DWH blowout in the Gulf of Mexico, and one that could be considerably more complex and difficult to combat given how much more hydrodynamic the FSC water column is compared to the Gulf of Mexico. These findings showed that crude oil alone does not act as an inducer for MOS formation in surface waters of the FSC, and that the addition of dispersant in the presence of oil appeared to be an important factor in triggering MOS formation, as observed in the CEWAF and CEWAF+N treatments. Even in the absence of crude oil, however, aggregates formed in the SW+D treatment and resembled those observed in the experiments of Kleindienst *et al.* (2015b) who used water from the Gulf of Mexico supplemented with the dispersant Corexit – a dispersant that was profusely used by BP on sea surface oil slicks and pumped directly at the leaking Macondo well-head during the DWH spill (National-Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011).

In both studies, these dispersant aggregates appeared white, gelatinous and viscous when handled. The findings of this study suggest that contrasting waters – i.e., the Gulf of Mexico and FSC – can lead to the formation of dispersant-induced aggregates displaying similar macroscopic characteristics. Since the use of dispersants in a marine setting is mainly as a contingency to combat oil spills, the SW+D treatment acted as a semi-control to test the effects of the dispersant on the microbial community in our experiments and is discussed below. The formation of MOS is likened to marine snow particles that are a crucial component of the biological pump in the ocean and defined as ‘hot spots’ for microbial activity (Long and Azam, 2001; Daly *et al.*, 2016) where there

exists a heightened level of enzyme activity and degradation rates compared to that in the seawater environment immediately surrounding these particles (Smith *et al.*, 1992). As reported by Giani *et al.* (2005), the formation of marine snow correlates with specific physical conditions (water column stratification, low mixing) and biological production patterns in the water column, such as nutrient concentrations, microbial production of TEP (Transparent Exopolymer Particles, discrete particles that can be visualised when stained with Alcian Blue) and EPS (Exopolymetric substances).

Furthermore, the formation and evolution of marine snow particles can vary considerably in terms of their size and content of mucilaginous matter, such as TEP and EPS (Giani *et al.*, 2005; Passow *et al.*, 2012) which are a matrix for marine snow formation (Passow, 2016). Here, the results showed that the presence of dispersant and crude oil (CEWAF treatment) yields MOS, but that oil alone (WAF treatment) does not. Furthermore, the addition of nutrients alone to seawater (SW+N treatment) triggers the formation of marine snow in surface waters of the FSC. Notably, nutrients amplified the abundance and size of MOS particles, as observed in the CEWAF+N treatment, and the structural integrity of these nutrient-aided MOS particles was more robust compared to that of their counterparts formed in the CEWAF treatment without added nutrients. Van Eenennaam *et al.* (2016) also described the formation of fragile MOS that easily falls apart when agitated, and that the bacteria associated with eukaryotic phytoplankton, principally through their production and release of EPS, enhances MOS formation. These findings indicate that microorganisms, in particular EPS-producing bacteria, play a key role in MOS formation, and that nutrients enhanced the activities of these organisms and yielded higher concentrations of EPS in the CEWAF+N. The hypothesis here is that EPS then interacted with crude oil and/or dispersant to form MOS, as previously observed (Gutierrez *et al.*, 2013). Some reports have also shown nutrient additions to seawater in influencing the formation of MOS (Kleindienst *et al.*, 2015a,b; Daly *et al.*, 2016 and references therein).

MiSeq sequencing was used to examine the bacterial taxa that were influenced by nutrients and potentially induced or upregulated the release of EPS and effected MOS formation in the CEWAF+N treatment. Hitherto, the only published study to have examined this type of community analysis for MOS was reported by Arnosti *et al.* (2016) who used Sanger sequencing of clone libraries to analyze the bacterial community associated with MOS that formed in roller-bottle experiments with sea surface waters

from the Gulf of Mexico. As in this present study, Arnosti *et al.* (2016) showed their MOS aggregates harboured a bacterial community composition that was distinctly different from that in the surrounding seawater. The MOS aggregates from the Gulf of Mexico were primarily composed of oil-degrading (*Cycloclasticus*, *Marinobacter*) and EPS-producing (*Halomonas*) bacteria, including diverse members of the order *Rhodobacterales* (principally within the family *Rhodobacteraceae*). This corroborates my results with FSC surface waters where the enrichment of these taxa on MOS formed in the CEWAF+N treatment was identified, as well as other taxa with recognized oil degrading (*Alcanivorax*, *Thalassolituus*, *Thalassospira*) and EPS-producing (*Pseudoalteromonas*, *Alteromonas*, *Vibrio*, *Cobetia*) qualities. This enrichment of bacterial taxa, which specialize in oil-degradation and EPS production, is consistent with the reduction in  $\alpha$ -diversity observed in the water fractions of this study over the course of the incubations over time (Figures 2.7 and 2.8). Kleindienst *et al.* (2015b) used catalyzed reporter deposition in combination with fluorescence *in situ* hybridization (CARD-FISH) to analyze MOS aggregates from their CEWAF+N treatments with Gulf of Mexico seawater and found the aggregates were dominated by members of the class *Gammaproteobacteria*, including the order *Alteromonadales*, and in particular members of the genus *Colwellia*, hence suggesting that *Colwellia* may play an important role in MOS formation in the presence of dispersants. During incubations with uncontaminated deep water samples collected during the active phase of the Gulf oil spill, Baelum *et al.* (2012) also reported the formation of MOS which was also dominated by members most closely related to *Colwellia*. Conversely, *Colwellia* contributed 0.01% abundance to MOS that formed in our CEWAF+N treatments by week 4 (T<sub>4</sub>), and the abundance of these organisms decreased sharply within week 1 in all the treatments amended with dispersant and/or crude oil. Hence, these contrasting water bodies of the Atlantic region (i.e., the Gulf of Mexico vs FSC) differ with respect to the bacterial taxa associated with MOS, and potentially also its formation and fate.

Interestingly, the most dominant organisms associated with MOS were members of the genus *Psychrobacter* (48.5% of total community reads from T<sub>4</sub> samples), which is a genus recognized for cold-tolerance – some have been isolated from permafrost and Antarctic waters – and reported to produce EPS (Leiye *et al.*, 2016). These organisms have also been found in waters contaminated with crude oil in the Arctic (Deppe *et al.*, 2005) and Southern Ocean (Prabakaran *et al.*, 2007), hence suggesting putative hydrocarbon-degrading qualities. *Psychrobacter* has not previously been reported

associated with MOS, and based on its dominant abundance of the total MOS-associated bacterial community these organisms may be an important contributor to MOS formation in surface waters of the FSC and/or the degradation of oil droplets associated with these aggregates. Although, further work will be needed to better elucidate this. It is posited that the collective community of opportunistic heterotrophs associated with MOS contributes two key roles. The first is in the formation of MOS, which was hypothesized to be mediated by EPS of organisms such as *Pseudoalteromonas* and *Alteromonas* that were abundant taxa associated with MOS at the initial stages of its formation (T<sub>2</sub>) in our experiments. The second is to the degradation of hydrocarbons within crude oil droplets entrained within the amorphous ‘net-like’ scaffolding of MOS. Here, it is hypothesized that hydrocarbon-degradation rates are markedly higher on MOS aggregates compared to in the surrounding seawater medium. This is supported by high rates of lipase hydrolysis activity detected on MOS aggregates formed in roller bottle experiments with surface seawater collected from the Gulf of Mexico during the DWH oil spill (Ziervogel *et al.*, 2012). The enrichment of obligate hydrocarbonoclastic bacteria on MOS, such as members of the genus *Alcanivorax* (33–42% relative abundance of the total MOS community) identified in our experiments, indicates MOS as a niche environment where oil biodegradation activities may be significantly elevated compared to that in the surrounding seawater environment. By week 4 the *Alcanivorax* population associated with MOS had decreased to <3% of the total community, suggesting that the bulk of the *n*-alkane hydrocarbons, which these organisms preferentially use as carbon substrates, had become sufficiently depleted on the MOS aggregates. This assumes these organisms had detached from the MOS aggregates to find new sources of utilizable hydrocarbons, which corroborates with the observed increase in their relative abundance in the seawater environment surrounding these aggregates.

Considering that MOS had already been observed on surface waters of the Gulf of Mexico during the DWH oil spill before BP had begun their operation of spraying tonnes of dispersants (Passow *et al.*, 2012), and laboratory roller-bottle experiments without added dispersants showed the rapid formation of MOS (Ziervogel *et al.*, 2012; Gutierrez *et al.*, 2013), MOS formation is very likely a biologically driven process. Halomonads, in particular, are commonly linked with the production of large quantities of EPS (Quesada *et al.*, 1994; Béjar *et al.*, 1998; Calvo *et al.*, 1998, 2002; Arias *et al.*, 2003; Gutierrez *et al.*, 2007), and like for many other EPS-producing marine bacteria (e.g., *Alteromonas*, *Pseudoalteromonas*), they can contribute large quantities of EPS to



the total DOM pool in the ocean (Azam, 1998). In fact, a large fraction of the DOM in the ocean is of glycoprotein in composition (Long and Azam, 1996; Verdugo *et al.*, 2004), which is consistent with the composition of marine bacterial EPS (Mancuso Nichols *et al.*, 2004; Gutierrez *et al.*, 2007; Hassler *et al.*, 2011). This concurs with my observation of MOS aggregates under the microscope after staining with AB or CBBG, which revealed marine snow formed in the SW+N treatments and MOS formed in the CEWAF and CEWAF+N treatments is largely composed of glycoprotein, and is evidence that it is of biogenic (likely bacterial) origin.

It has been suggested that MOS formation is initiated via the physicochemical interaction between oil droplets, microbial cells and biopolymer – the latter likely of microbial origin (Passow *et al.*, 2012). These results showed that the presence of a dispersant (Superdispersant-25) enhances MOS formation, as observed in the CEWAF treatment and reported elsewhere using the dispersant Corexit that was used at DWH (Fu *et al.*, 2014). Nutrients were, however, found to amplify the abundance and size of MOS, as observed in the CEWAF+N treatment. However, the fact that marine snow was formed in the SW+N treatment, without any added dispersant, suggests that MOS formation is indeed a biologically driven process that likely involves endogenous DOM in seawater (in the form of TEP and EPS) and which is likely enhanced by the *de novo* synthesis of EPS by EPS-producing bacteria. This is supported by the diversity of EPS-producing taxa that were identified enriched on MOS that formed in the CEWAF+N treatment. Correlating this to the Gulf of Mexico environment where profuse quantities of MOS were observed during the DWH oil spill, Lin and Guo (2015) found elevated levels of dilute-HCL-resistant polysaccharides (HR-PCHO) and total dissolved carbohydrates-to-dissolved organic carbon (TCHO/DOC) ratios at some sampling stations. This likely resulted from enhanced microbial production of EPS due to the presence of oil components and nutrient inputs from the Mississippi river (Muschenheim and Lee, 2002; Khelifa *et al.*, 2005).

The CEWAF and CEWAF+N treatments simulated the application of a UK-approved dispersant (Superdispersant-25) and of nutrients – approaches that are often used as a bioremediation strategy for combatting marine oil spills – to investigate the microbial response in surface waters of the FSC. Although measurements for nutrient concentrations were not conducted, the observed increase in prokaryotic cell abundance, as well as significantly greater  $\alpha$ - and  $\beta$ -diversities, in the SW+N treatments is indicative

that nutrients are a significant limiting factor in surface waters of the FSC. In support of this, experimental studies in the North Atlantic have shown that bacterial growth can be restricted by the availability of  $\text{PO}_4^{3-}$  (Cotner *et al.*, 1997; Rivkin and Anderson, 1997; Caron *et al.*, 2000). Interestingly, Kleindienst *et al.* (2015b) observed highest prokaryotic cell abundances in WAF treatments by the end of their experiments, whereas this study reported highest abundances in the CEWAF+N treatments. This difference across these two studies may be explained by differences in endogenous concentrations of nutrients in the Gulf of Mexico compared to in the FSC that could support growth without addition of an exogenous carbon source (e.g., crude oil or dispersant). Differences in crude oil constituents and their solubility, as well as concentrations of labile/semi-labile DOM between these studies should also be considered. Higher cell abundances in the CEWAF treatments were also measured compared to in the WAF treatments. Taken collectively, these results suggest that the presence of dispersant, and particularly added nutrients, stimulate microbial growth in FSC surface waters when contaminated with crude oil. Whether any microbial group was able to degrade and grow on the dispersant used in this study (Superdispersant-25) remains to be investigated.

The microbial community analysis of FSC surface waters indicated that members of the order *Alteromonadales* and *Rhodobacterales* constituted the dominant proportion (96%) of total sequence reads – lineages which are consistently found and often in high relative abundance in surface waters of the Gulf of Mexico (Yang *et al.*, 2016) and open-Atlantic ocean (Swan *et al.*, 2011). However, the exception was a lack of representation by the SAR11 clade, which is a major group that is commonly found in pelagic waters (Morris *et al.*, 2002) and, quite possibly, because this group has been shown to be susceptible to oil pollution (Lanfranconi *et al.*, 2010; Chronopoulou *et al.*, 2015). Whilst it is planned to analyze whether the surface waters of the FSC are contaminated with hydrocarbons, as might be likely due to the prevalent oil extraction activities occurring in these waters and in those of the adjacent North Sea, the presence of hydrocarbon contaminants could explain the fact that in our sequencing libraries these organisms are not present. *Colwellia* is a genus of psychrophilic marine heterotrophic generalists, which expectedly was found in the cold surface waters of the FSC, but atypically in quite high relative abundance. Unlike the rapid colonization of these organisms in sea surface oil slicks and subsurface oil plume in the Gulf of Mexico during the DWH spill (Redmond and Valentine, 2012; Yang *et al.*, 2016), the dramatic decline of *Colwellia* in our experiments amended with dispersant, crude oil or both suggests that these organisms

may too be susceptible to hydrocarbons in FSC surface waters and to synthetic dispersants, such as Superdispersant-25. Their rapid reduction in the SW+N treatment, however, suggests that these organisms may also suffer a competitive disadvantage to other members of the community during periods of spiked nutrient influxes. *Colwellia* in the surface waters of the FSC may be physiologically inclined as strict oligotrophs. This is in contrast to certain oligotypes of *Colwellia* that were identified in the Gulf of Mexico with a predilection for degrading and growing on the dispersant Corexit and crude oil (Kleindienst *et al.*, 2015b). Of further interest, surface waters of the FSC contained a dominance of *Sulfitobacter* (up to 28%), which is a sulfite-oxidizing member of the *Alphaproteobacteria* within the *Roseobacter* clade (Buchan *et al.*, 2005). The abundance of these organisms dramatically fell and was sustained at low levels (often < 2%) by the presence of either exogenous nutrients or crude oil. However, in the presence of dispersant (+/- crude oil and nutrients), an initial dramatic reduction in their abundance was followed by their recovery to abundances >5% and as high as 50%. Since Superdispersant-25 is a sulphur-containing dispersant, it is likely that certain members of the *Sulfitobacter* community sustained a relative high abundance in these dispersant-amended treatments because they were capable of feeding on the sulfur constituent as an energy source.

The presence, albeit in relative low abundances (<0.6%), of obligate hydrocarbonoclastic bacteria (*Alcanivorax*, *Cycloclasticus*, *Oleispira*, *Thalassolituus*) – organisms that are recognized as key players in the biodegradation of crude oil and its refined petrochemical products in the marine environment (Yakimov *et al.*, 2007) – was not unexpected, and included representation of the ‘generalist’ oil-degrader *Marinobacter*. These organisms are typically found at background levels in the global ocean (Yakimov *et al.*, 2007). With the exception of *Oleispira*, the intermittent (1 week) or sustained (over several weeks) bloom of these organisms in the CEWAF and/or CEWAF+N treatments is reminiscent of their strong enrichment in oil-impacted environments where they can be expected to increase in numbers from near undetectable levels.

Other taxa that were also strongly selected for in these treatments included *Halomonas*, *Alteromonas* and *Pseudoalteromonas* – genera that contain members with reported hydrocarbon-degrading capabilities, though are more commonly associated with producing EPS (Béjar *et al.*, 1998; Calvo *et al.*, 1998, 2002; Arias *et al.*,

2003; Mancuso Nichols *et al.*, 2004; Bhaskar and Bhosle, 2005; Gutierrez *et al.*, 2007, 2008, 2009, 2013). Interestingly, a study that investigated the response of pelagic bacterial communities to crude oil in the North Sea showed that the most dominant responder was *Pseudoalteromonas* (10-fold enrichment), with practically no detection for any of the obligate hydrocarbonoclastic taxa; however, denaturing gradient gel electrophoresis (DGGE) of the bacterial 16S rRNA gene was used for analyzing microbial community profiles in this study which, based on its limited coverage for capturing near total diversity, will likely have missed less abundant taxa (Chronopoulou *et al.*, 2015). Nonetheless, this highlights how different water bodies, even those adjacent to each other at the same or proximal latitude, can yield differential microbial community responses to crude oil contamination, which may be attributed to, though not always entirely, to a predilection of certain taxa to hydrocarbons.

Interestingly, a short, but strong enrichment in the CEWAF and CEWAF+N treatments for members of the *Vibrionales* – principally the genus *Vibrio* – revealed that these organisms may participate in the degradation of crude oil in FSC surface waters. The enrichment of these organisms is not frequently observed at contaminated sites in the marine environment, although there are snippets in the literature reporting on the enrichment of these organisms by crude oil. For example, members of the *Vibrionales* were found enriched in beach sands of the Gulf coast that had become contaminated with Macondo oil from the DWH spill, and several oil-degrading *Vibrio* spp. were isolated and found to degrade hydrocarbons (Kostka *et al.*, 2011). Also, a 91-fold increase in the relative abundance of *Vibrionales* was detected in oil contaminated sea surface oil-slick water samples from DWH when incubated to develop anaerobically (Gutierrez *et al.*, 2016). An analysis of the genomes of several *Vibrio* species found these organism capable of metabolizing hydrocarbons, including PAHs (Grimes *et al.*, 2009). Further work will be needed to fully understand the hydrocarbon-degrading potential and role of these organisms in the FSC.

This study highlights the importance for the application of dispersants and/or nutrient-amendments in MOS formation in the event of an oil spill in the FSC. I also identified oil-degrading and EPS-producing bacteria associated with MOS, and that crude oil alone does not yield MOS in these waters. It is noted that the seawater used in this study was obtained from one seasonal period of the year (the winter of 2015) and that

further work would be needed to explore MOS formation in waters collected during other seasons in order to provide a more conceptual understanding of this process given the unpredictability of when an oil spill might occur in the FSC. It has also been demonstrated that surface waters of the FSC harbour communities of hydrocarbonoclastic bacteria that positively respond to crude oil contamination, and that amending these waters with dispersant and/or nutrients could stimulate microbial community activities. Based on these findings, such approaches should be considered in bioremediation strategies in the event of a major oil spill in this region of the northeast Atlantic, although further instigative work to assess this is warranted. Essentially, the influence of dispersants on oil-degrading bacteria remains poorly understood and requires further investigation using different types of dispersants and evaluation across different water bodies. These findings on MOS formation and the microbial response to oil in FSC surface waters mirror those observed following the DWH disaster and hence underscore their broad relevance.

## **2.7. Conclusion**

Marine oil snow is possibly the most important mechanism by which oil reaches the seafloor in the event of a spill at sea. This study shows that in the event of an oil spill in the FSC, the use of dispersants would likely lead to the formation of MOS and trigger a subsurface “dirty blizzard,” reminiscent to that during the DWH oil spill where a large proportion of sea surface oil ended up on the seafloor. In the absence of dispersant applications, the majority of surface oil is likely to remain at the sea surface. Bacterial communities seem to respond to the presence of oil and/or dispersant and this will have a direct and /or indirect repercussion on the rest of organisms involved in the marine ecosystem. Hence, any research conducted to evaluate crude oil impacts to benthic ecosystems in the FSC would need to take into account the physicochemical state of the oil presented in the form of MOS aggregates – direct exposure of sediment samples or cores to crude oil for such investigations would be unrealistic. This study also showed that MOS particles formed with FSC surface seawater harbour rich communities of prokaryotes, including oil-degrading bacteria, potentially acting as ‘hot spots’ where a heightened level of oil biodegradation occurs.



**Chapter III: Chemical dispersant enhances  
microbial exopolymer (EPS) production and  
formation of marine oil/dispersant snow in  
surface waters of the subarctic northeast Atlantic**

### **3.1. Overview**

In this study it is reported that during exposure of FSC seawater to a chemical dispersant, whether in the presence/absence of crude oil, the dispersant stimulates the production of significant quantities of EPS that would serve as a key building block in the formation of MOS. This response is likely conferred via de-novo synthesis of EPS by natural communities of bacteria. The formation of marine dispersant snow (MDS) as a product of adding chemical dispersants to seawater is also described. In this Chapter, the sections of Results and Discussion are joint for a better explanation and understanding for the reader. This chapter has been published in the journal *Frontiers in Microbiology* (see List of Publications).

### **3.2. Hypothesis**

It has been shown in Chapter II that the presence of dispersant seems to enhance MOS formation and EPS- producers and oil-degrading bacteria communities develop in its presence. This suggests the possibility that EPS could be one of the factors regulating MOS formation.

### **3.3. Introduction**

It is estimated that EPS (extracellular polymeric substances) form an organic carbon pool of 70 Pg C in the ocean (Verdugo *et al.*, 2004) and which exists as dissolved, colloidal and gel particles in seawater (Santschi *et al.*, 2003; Verdugo *et al.*, 2004). A wide variety of microorganisms produce and secrete EPS in the marine environment and which contributes significantly to the dissolved organic carbon (DOC) in the global ocean water column – ca.10-25% of total oceanic dissolved organic matter (Verdugo, 1994; Aluwihare *et al.* 1997). Compared to EPS produced by freshwater/marine eukaryotic phytoplankton and non-marine bacteria, EPS produced by marine bacteria generally contain higher levels of uronic acids, notably D-glucuronic and D-galacturonic acids (Kennedy and Sutherland, 1987). These acidic sugars can render these macromolecules polyanionic (negatively charged) and ‘sticky’, and consequently confer them with the ability to form aggregates, such as marine snow and transparent exopolymer particles (TEP) (Wotton, 2004a). In the marine environment, APS (Acidic polysaccharide substances) is found dissolved in the water column and as a major component of TEP, as well as involved as a protective external layer of some microorganisms (Thornton, 2009). As one of the most common types of EPS produced by bacteria and eukaryotic phytoplankton (Passow and Alldredge, 1994;



Stoderegger and Herndl, 1999), APS is known to contribute to the formation of marine snow (Alldredge *et al.*, 1993), and recent work has shown it to be implicated also in the formation of MOS (Gutierrez *et al.*, 2013; Gutierrez *et al.*, 2018; Passow *et al.*, 2012; Passow, 2016).

To-date, observations of marine dispersant snow (MDS) have only been documented in two reports employing laboratory-based experiments (Kleindienst *et al.*, 2015a; Chapter II of this thesis), though their potential to form during the application of dispersants at sea during an oil spill warrants attention. Whilst the mechanism(s) involved in MOS formation still remain largely unresolved, the prevailing evidence implicates EPS and dispersants, either independently or in combination, as key agents in this process. Here, this study leads to the investigation of the role of EPS (APS proxy) in MOS formation in surface waters of a subarctic region in the northeast Atlantic, and how this process might be influenced by chemical dispersants. MDS formation and an analysis of the communities of bacteria associated with these particles were also assessed and compared to those with MOS.

### **3.4. Materials and Methods**

#### **3.4.1. Field samples**

During a research cruise on RV *Scotia* on 11 October 2017, sea surface water samples were collected from a depth of 5 m at a subarctic northeast Atlantic region called the FSC (60°38.120' N, 4°54.030' W) – *in-situ* temp. 8.7°C. This sampling site is exactly the same than the one studied in section 2.3.1 in Chapter II.

#### **3.4.2. Water-accommodated fractions**

The method used here is the same as the one used in section 2.3.2 of Chapter II. A 800 mL volume of filtered seawater was amended with 140.8 mL of pre-filtered (0.22 µm) Schiehallion crude oil (provided by BP). To prepare chemically-enhanced WAF (CEWAF) medium, 800 mL of filtered seawater was amended with 140.8 mL of filtered Schiehallion crude oil and 14.08 mL of Superdispersant-25 (provided by Oil Slick Dispersants Ltd.). Seawater amended with only dispersant comprised 800 mL of the filter-sterile seawater and 14.08 mL of Superdispersant-25. The effective dilution of the dispersant in the seawater treatments (dispersant-to-oil ratio, v/v) was 1:10, which is a dilution recommended by the oil and gas industry

(<https://www.gov.uk/government/publications/marine-pollution-contingency-plan>,

Approved oil spill treatment products, Government UK, July 2016).

The various mixtures of sterile seawater (SW) amended with oil (WAF), oil+dispersant (CEWAF) or solely dispersant (SW+D) were mixed on a rotary magnetic stirrer at 140 rpm for 48 h at 7°C in the dark in clean sterile (acid-washed) 1 L glass bottles. The mixtures were then allowed to stand for 1 h prior to transferring the aqueous phases into clean Teflon-lined screw-capped glass tubes whilst avoiding the non-dispersed/non-solubilized oil. The SW and these WAF, CEWAF and SW+D solutions were then stored at 4°C and used within 48 h for the microcosm experiments. For treatments containing nutrients – i.e., seawater+nutrients (SW+N) and CEWAF+nutrients (CEWAF+N) – the solutions were amended with 10 µM ammonium chloride, 10 µM sodium nitrate and 1 µM potassium phosphate (final concentrations).

### **3.4.3. Microcosm setup and sampling**

To examine the microbial response and formation of MOS and MDS in sea surface waters of the FSC when exposed to crude oil, dispersant and/or nutrients, a roller-bottle design was used, as previously described (Chapter II of this thesis). This roller bottle setup has been used widely to investigate marine snow (Shanks and Edmondson, 1989) and MOS (Passow, 2012; Kleindienst *et al.*, 2015a) formation as it simulates natural sea surface/pelagic conditions in a laboratory setting (Jackson, 1994). For this experiment, four microcosm treatments (WAF, SW+D, CEWAF, CEWAF+N) were setup, each prepared using 500 mL Pyrex® glass bottles that were maintained at constant rotating gentle motion using a roller table device. Each treatment was run in triplicate and comprised of 85.5 mL of filter-sterile WAF, dispersant-only, CEWAF or CEWAF+N added to 300 mL of unfiltered natural seawater from the FSC. In addition, two oil/dispersant untreated controls were setup and run in parallel: one comprised seawater alone (SW), and the second of seawater with only nutrients added (SW+N). Treatments and controls were each established in triplicates and incubated at 8°C (*in-situ* sea surface temp. in the FSC at the time of sampling) and in the dark at a rotation speed of 15 rpm.

All bottles for each treatment and the controls were sampled, taking extreme care in order to avoid disrupting aggregates that may have formed. Sampling was performed at five time points over the course of 4 weeks: at the beginning of the experiment ( $T_0$ ), after 1 week ( $T_1$ ), after 2 weeks ( $T_2$ ), after 3 weeks ( $T_3$ ), and after 4 weeks ( $T_4$ ). At each

sampling time, each bottle was placed gently in an upright position to capture a photographic record of the contents within (e.g. change in color and formation of MOS or MDS). When formed, sub-samples of MOS or MDS aggregates were carefully withdrawn using pre-sterilized glass Pasteur pipettes and transferred to 1.5-mL microcentrifuge tubes for staining with the cationic copper phthalocyanine dye Alcian Blue (AB) at pH 2.5 (Alldredge *et al.*, 1993), or with the amino acid-specific dye Coomassie Brilliant Blue (CBBG) at pH 7.4 (Long and Azam, 1996). AB is used for staining acidic sugars of EPS or transparent exopolymer particles (TEP) in seawater, whereas CBBG is used for staining the proteinaceous component of these polymeric substances. Following staining, the aggregates were washed by transferring them through several droplets of sterile water prior to their examination under the light microscope. To directly examine the prokaryotic community under the microscope, MOS and MDS particles were also stained with acridine orange (AO) (Francisc *et al.*, 1973) for imaging with a FITC filter on a Zeiss Axioscope epifluorescence microscopy (Carl Zeiss, Germany). Only the light and the contrast of the picture taken were modified with Image J software (Rueden *et al.*, 2017). Moreover, MOS or MDS aggregates were also sub-sampled during these experiments for DNA extraction and analysis of their associated bacterial community (described below). Observations of all treatments and controls were also recorded for changes in turbidity and/or emulsion formation.

#### **3.4.4. Genomic DNA extraction and barcoded-amplicon sequencing and analysis**

DNA was extracted from MDS and MOS aggregates according to the method of Tillett and Neilan (2000). Purified DNA was stored at  $-20^{\circ}\text{C}$  for subsequent molecular analysis. Barcoded 16S rRNA gene MiSeq sequencing, targeting the V4 hypervariable region, was employed to analyze the bacterial community of the different aggregates over the 4-week duration of the experiments at time points T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>4</sub>. The 16S rRNA gene was amplified in duplicate 25  $\mu\text{l}$  reactions, and replicates were subsequently pooled to increase PCR product yield. Each reaction comprised 10.5  $\mu\text{l}$  of molecular biology grade water, 12.5  $\mu\text{l}$  of Platinum Hot Start Master Mix, 0.5  $\mu\text{l}$  each of 10  $\mu\text{M}$  forward (515f) and reverse (806r) primers, and 1  $\mu\text{l}$  of template DNA. The primers used were 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACNVGGGTWTCTAAT) (Caporaso *et al.*, 2011, 2012). Both primers had Illumina MiSeq overhangs and unique golay barcodes added to the 5' ends. All PCR products were purified by GFX PCR purification (#GE28-9034-70, Sigma, UK). All samples were sequenced via the Illumina

MiSeq platform (Illumina 2 x 250 V.2 kit) at the Edinburgh Genomics sequencing facility (Edinburgh University, UK), and raw sequences were demultiplexed prior to receipt at our laboratory. All sequences were deposited in the SRA repository under accession numbers SAMN10417097 to SAMN10417120.

Subsequent processing of the Illumina sequence data was performed using the DADA2 package as wrapped in QIIME2 (Callahan *et al.*, 2016). This step was done by Stephen Summers at Heriot Watt University. In brief, paired end Illumina In review 10 reads (Phred 33) were combined to form contiguous sequences. A fragment cut-off of 220 bp was established to maintain quality. These contigs were examined for low quality phred scores and any identified chimeric sequences were removed. All quality-approved sequences were compared on a single nucleotide resolution and the resulting single nucleotide variants (SNVs) were identified using the Green Genes database of 16S rRNA gene taxonomy. Alpha- and beta-diversity indices were collected for the individual samples and treatment types, respectively. For alpha-diversity analysis, sampling was standardized to a ceiling of 44,800 sequences per sample, and rarefaction and ordination analyses both utilized this standardization. All sequences were deposited in the SRA repository under accession number SAMN10417097 to SAMN10417120.

### **3.4.5. EPS extraction and analysis**

Alcian blue is a cationic dye that binds with the carboxyl (COO<sup>-</sup>) and half-ester sulfate (\_OSO<sub>3</sub>\_) groups of APS, but does not complex with neutral sugars (Ramus 1977; Passow and Alldredge, 1995). Hence, it was used here to quantify APS as a proxy of EPS concentrations in the microcosm treatments – this analysis was conducted in collaboration with St. Andrews University in the laboratory of Prof. David Paterson and carried out by Cindy Chen following the protocol described in Chen *et al.*, 2017. For this, a stock solution of Alcian Blue (GX8) was prepared (0.02% w/v final concentration) in distilled water containing 0.06% v/v acetic acid (analytical grade). The solution was sonicated for 15 min to disaggregate particulates of the Alcian Blue, then stirred for 30 min with a magnetic stirrer, and the solution stored at 4°C. Prior to use, the Alcian Blue solution was several times passed through 0.45 µm filters to remove any dye coagulation. The stability of the final solution was determined by a constant absorbance reading (602 nm) between sequential filtrations. A standard curve was produced using xanthan gum as a proxy for natural APS (after Hope, 2016) and total APS concentrations were obtained as xanthan equivalents (µg X eq. mL<sup>-1</sup>). In review, 11 aliquots for APS analysis (1.5 ml) were

transferred to 3 mL glass centrifuge tubes. The samples were vortexed for 30 sec, centrifuged (2500 rpm; 20 min), and then 1 mL of pre filtered Alcian Blue solution was added to each sample. The Alcian-sample mixture was resuspended and vortexed for 30 sec to ensure an irreversible bond formed between the Alcian Blue and APS. The samples were then centrifuged (2500 rpm; 20 min) to release unbound dye into the supernatant, which was carefully removed. The remaining dyed pellet was rinsed with distilled water and centrifuged again, and this was repeated until no excess dye was released (cf 3 washes). The remaining stained pellets were treated with 6 mL of 80% 258 (v/v) sulphuric acid, suspended and sonicated for 15 min and then vortexed for 30 sec to obtain a homogenous solution. The samples were gently agitated 2-3 times during an incubation period of 2 h (color stable for 2-20 h), to release any oxygen bubbles. Sulphuric acid disassociated the bound dye from the pellet, resulting in a gradation of green solutions depending on the concentration of APS in the samples. The absorbance of each sample was measured spectrophotometrically (787 nm, Biomate 5 spectrophotometer).

#### **3.4.6. Statistical analyses**

Relative abundances of sequences obtained using MiSeq were compared between treatments by PERMANOVA, at a level 5 taxonomy (family). EPS concentrations, based on APS measurements, were compared between the different treatments and timeline using Analysis of Variance (ANOVA) followed by a Post-Hoc Tukey test. All data was log transformed to meet the assumptions of parametric analysis.

### **3.5. Results and Discussion**

#### **3.5.1. Formation and chemical composition of MOS**

The formation of MOS was observed in the CEWAF and CEWAF+N treatments within 6 days from the commencement of these experiments. Initially, the MOS particles appeared small (0.5–1 mm) and dark brown, and by the end of these incubations (at day 28) they had increased in size (2–5 mm) and remained floating at the surface (Figure 3.1A). They did not settle to the bottom of the bottles at any time over the 4-week duration of these experiments. At the termination of these experiments (T<sub>4</sub>), the particles appeared somewhat lighter in colour, suggesting that the oil hydrocarbons entrained within the MOS particles had become, at least partially, degraded. As previously reported (see Chapter II of this thesis), the presence of the chemical dispersant Superdispersant-25 was a key component to yielding MOS, and the presence of nutrients magnified this response – i.e. the abundance of MOS particles in the CEWAF+N treatment was much higher than

in the CEWAF treatment without nutrients. MOS also formed in the CEWAF treatment, but because they were comparatively smaller in size and consequently difficult to handle, they were not further analyzed. It is noted that this dispersant-induced formation of MOS is reproducible across seasons of the year, as this response was demonstrated in roller bottle experiments performed at our laboratory using surface seawater collected from the FSC in the winter of 2015 (see Chapter II of this thesis). Conversely, the formation of MOS in roller bottle experiments was not observed using surface seawater collected from the FSC during the spring of 2015 when exposed to solely Schiehallion crude oil and without chemical dispersants (results not shown). Corroborating with my previous results (Chapter II), the formation of MOS was not observed in the WAF treatment (no dispersant added), whereas the formation of marine snow (without oil) in the SW+N treatment was observed. These results add to a growing body of evidence indicating that in the event of an oil spill at sea, the application of chemical dispersants is important in triggering the formation of MOS (Fu *et al.*, 2014; Kleindienst *et al.*, 2015a; Doyle *et al.*, 2018). However, it should be noted that under certain conditions, which remain unresolved, MOS can form in the absence of a chemical dispersant. Passow *et al.* (2017), for example, showed that diatoms resulted in increased MOS formation, whereas its formation in the presence of diatoms was inhibited when dispersant was added. In the absence of dispersant application for any specific oceanic region, any number of environmental factors (e.g. sea surface and hydrodynamic variables, algal/bacterial community profile, DOC/EPS concentrations in seawater etc.) could be implicated in initiating MOS formation during an oil spill. Our results show that in surface waters of the FSC – a subarctic region of the northeast Atlantic – the application of a chemical dispersant does result in the formation of MOS and MDS.

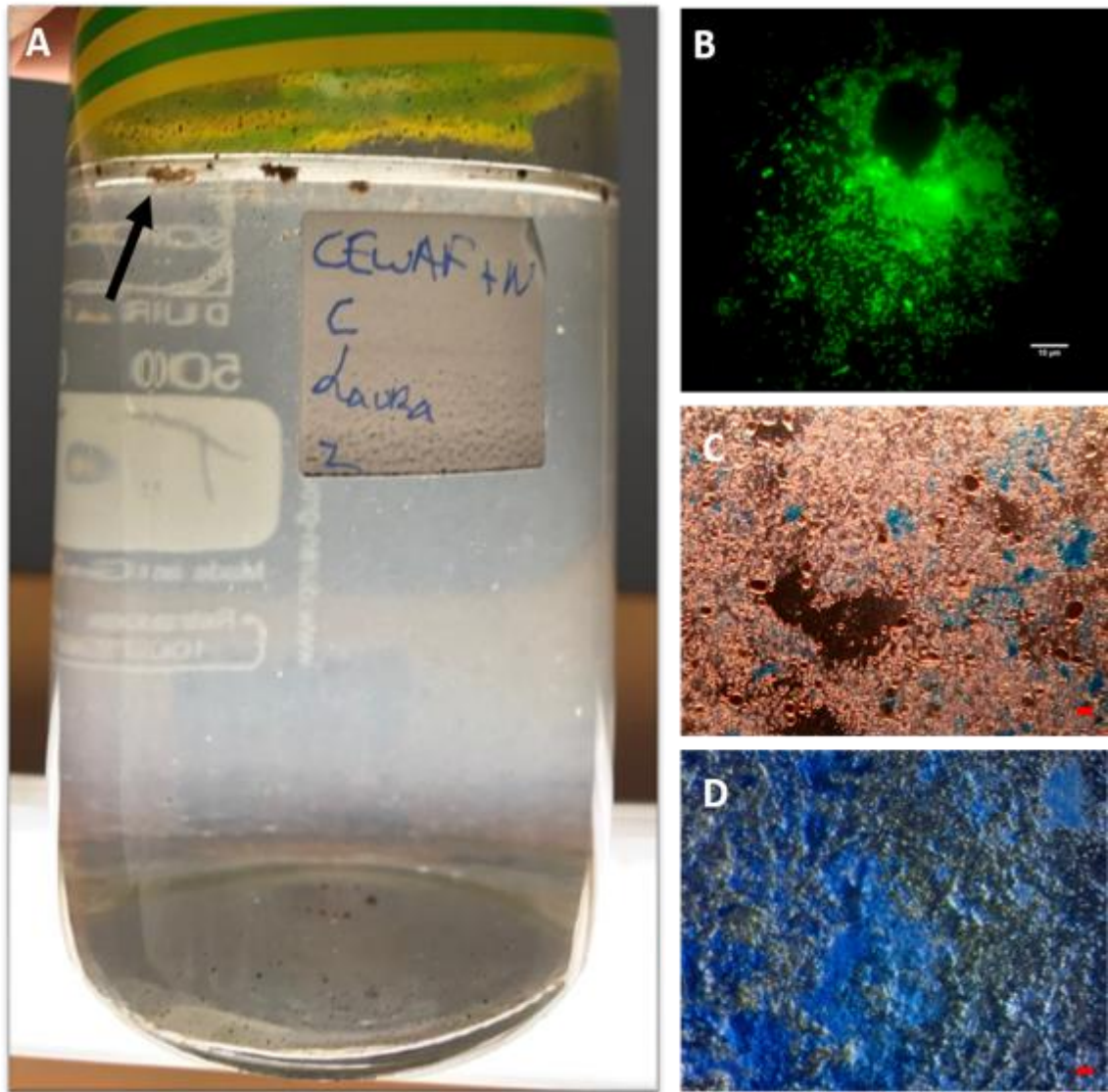
When observed under the epifluorescence microscope with AO staining, MOS aggregates from the CEWAF and CEWAF+N treatments were heavily enriched with prokaryotic cells (Figure 3.1B) – some aggregates were also observed to contain oil droplets (not shown). When viewed under the light microscope with the aid of dark field illumination, these aggregates appeared to partially stain with CBBG (Figure 3.1C), whereas they predominately stained with AB (Figure 3.1D). This provides evidence that MOS aggregates formed in the presence of the dispersant, oil and with/without nutrients (i.e. the CEWAF and CEWAF+N treatments) were of glycoprotein composition, with a heavy compositional loading of EPS. Previous work assessing MOS formation in waters of the Gulf of Mexico during the active phase of the DWH oil spill showed MOS

aggregates as highly susceptible to peptidase and  $\beta$ -glucosidase activities (Ziervogel *et al.*, 2012), which was indicative of their glycoprotein composition. As glycoproteins are a major component of marine bacterial EPS (Long and Azam, 1996; Verdugo *et al.*, 2004; Hassler *et al.* 2011; Mancuso Nichols *et al.*, 2004), these studies collectively point to marine bacteria as a source of these polymers and in playing a key role in MOS formation. The amino acid and peptide components comprising these bacterial glycoproteins can confer amphiphilic characteristics to these macromolecules (Verdugo *et al.*, 2004; Gutierrez *et al.*, 2009) and in turn allow them to associate with petrochemical hydrocarbons and crude oil droplets. Previous work assessing MOS formation in surface water samples collected from the Gulf of Mexico well after the DWH spill showed no formation of MOS (Ziervogel *et al.*, 2014). These experiments had been performed without any added chemical dispersant. It can be conjectured, therefore, that by September 2012 (ca. 2 years and 5 months after the onset of the DWH spill when these water samples were collected) there was either none, or insignificant concentrations, of the Corexit remaining in the Gulf water column. MOS, however, formed in roller bottle experiments performed by the same researchers when using seawater collected from the Gulf during the active phase of the DwH spill (Ziervogel *et al.*, 2012). Although no chemical dispersant had been added to those experiments, it is suspected that the seawater samples used by Ziervogel *et al.* (2012) contained Corexit since it had been applied in copious quantities on the sea surface and subsurface, and which would explain the observed formation of MOS and also supporting the role of chemical dispersants in this process. Taken collectively, these and other studies point to chemical dispersants as an important contributor to triggering MOS formation, and that nutrients enhance this response.

### **3.5.2. Formation and chemical composition of MDS**

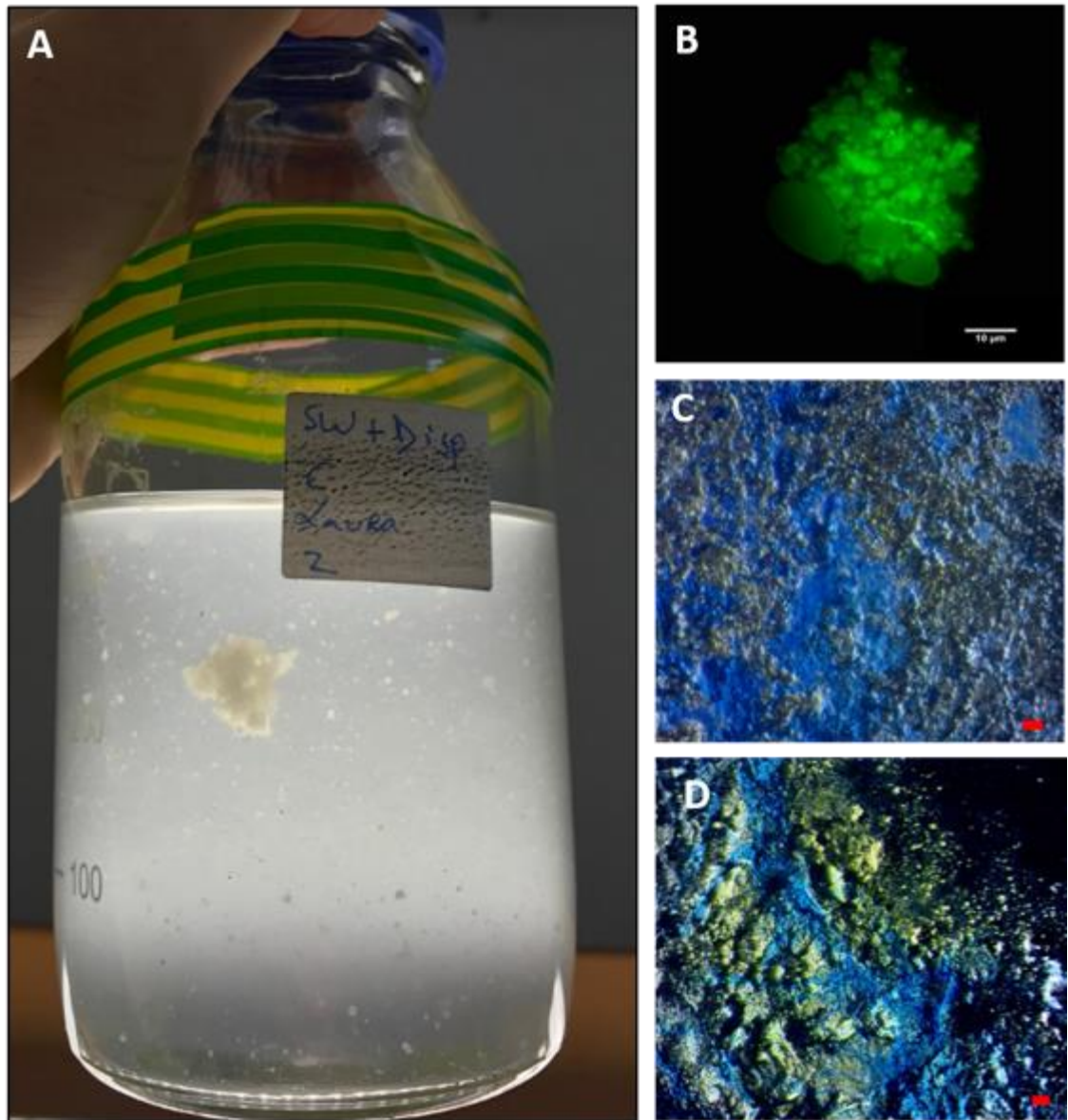
MDS formed within 3 days in the SW+D treatment only, appearing like ‘cotton wool’ and white to off-white in coloration (Figure 3.2A). A control showed that dispersant alone does not stain with CBBG nor AB. Over time, some of the particles grew in size (up to 0.5–1.5 mm by the end of the experiment), although many remained quite small (typically < 0.05mm); eg. the particles in treatments amended with the chemical dispersant (SW+D, CEWAF, CEWAF+N) got bigger in size overtime compared to treatments with no added dispersant (SW, SW+N, WAF). When observed under the epifluorescence microscope with AO staining, MDS aggregates from the SW+D treatments did not show high presence of prokaryotic cells (Figure 3.2B) – compared with

the MOS aggregates (Figure 3.1B). When viewed under the light microscope with the aid of dark field illumination, these aggregates appeared to be both strongly stained with CBBG (Figure 3.2C) and AB (Figure 3.2D).



**Figure 3.1.** MOS aggregates formed in the CEWAF+N treatment shown at 4 weeks floating on the surface (indicated by arrow) of the roller bottle (A). An aggregate from this treatment stained with acridine orange and viewed under the epifluorescence microscope with a FITC filter shows a rich community of prokaryotic cells (small green dots) (B). MOS stained with Coomassie Brilliant Blue G (C) and Alcian Blue (D). Bar 10 µm.



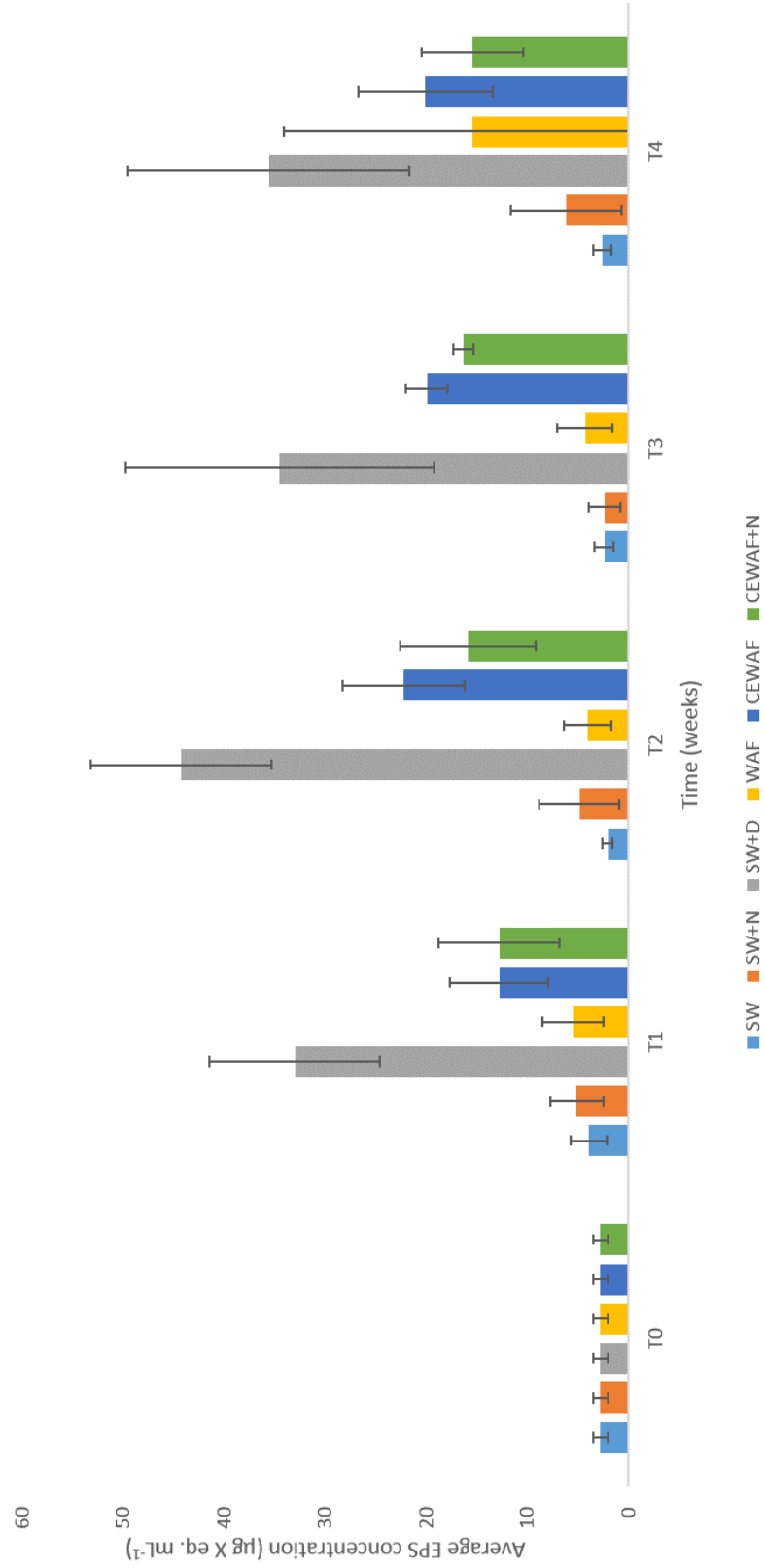


**Figure 3.2.** MDS aggregates formed in the SW+D treatment shown at 4 weeks (A). An aggregate from this treatment stained with acridine orange and viewed under the epifluorescence microscope with a FITC filter shows very few associated prokaryotic cells (small green dots), but an apparent abundance of dispersant globules/droplets (B). MDS stained with Coomassie Brilliant Blue G (C) and Alcian Blue (D). Bar, 10 µm.

Based on APS analysis (Figure 3.3), average EPS concentrations and their respective standard deviations across all treatments at the start of these experiments ( $T_0$ )

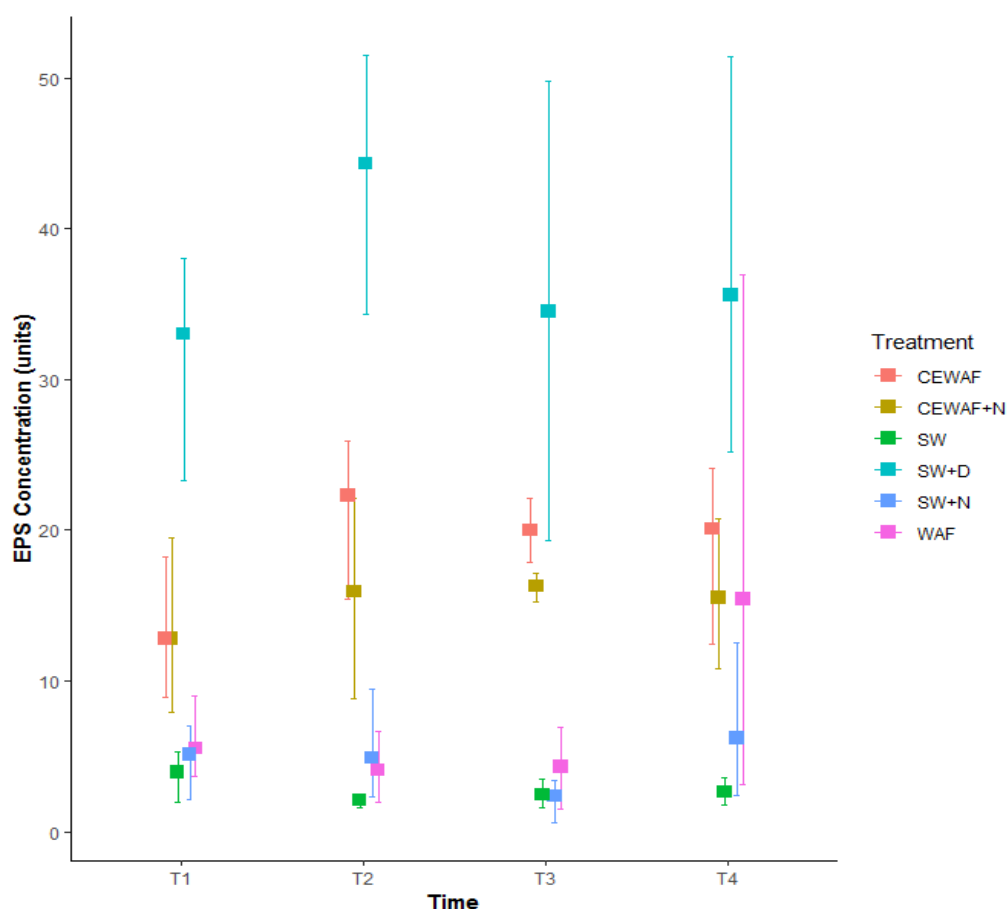
were  $2.7 \pm 0.7 \mu\text{g X eq. mL}^{-1}$ , which is concomitant with values reported in surface seawater of the subarctic and arctic (Hong *et al.*, 1997; Ramaiah *et al.*, 2001), and other sites such as the Gulf of Mexico (Thornton *et al.*, 2007). Within one week ( $T_1$ ), EPS concentrations increased by ca. 10-fold (to  $33.0 \pm 8.0 \mu\text{g X eq. mL}^{-1}$ ) in the SW+D treatment, and by ca. 4-fold (to  $13.0 \pm 5.0 \mu\text{g X eq. mL}^{-1}$ ) in the CEWAF and CEWAF+N treatments. Average concentrations of EPS in these treatments remained high over the remaining duration (weeks 2 to 4) of these experiments, at 33-45  $\mu\text{g X eq. mL}^{-1}$  in the SW+D treatment, and 15-20  $\mu\text{g X eq. mL}^{-1}$  in the CEWAF and CEWAF+N treatments. Statistical analysis showed significant differences between these treatments compared to the other three ones: SW, SW+N and WAF. In contrast, no significant change in EPS concentrations ( $p > 0.05$ ) were detected in the SW, SW+N and WAF treatments over the entire duration of these experiments, with concentrations averaging 3.0-5.5  $\mu\text{g X eq. mL}^{-1}$  the exception was the WAF treatment at week 4, but the increased EPS concentration measured here can be attributed to an outlier of the triplicate samples analyzed, as shown by the large error bar range.

In previous work (Chapter II of this thesis) and in this study, it was shown that chemical dispersants and nutrients enhance MOS formation, particularly in their size and abundance. Here, the fact that the presence of chemical dispersants can increase the concentration of EPS in seawater is also shown. Similarly, EPS consisting of proteins and polysaccharides was produced in laboratory experiments when exposing eukaryotic phytoplankton communities to chemical dispersants (Van Eenennaam *et al.*, 2016). Notably, in our experiments this phenomenon also occurred in the absence of crude oil (SW+D treatment) where this measured increase in EPS concentration was highest across the various treatments; although it was also significantly higher in the other dispersant-amended treatments (CEWAF, CEWAF+N) compared to in the treatments without dispersant. Furthermore, it should also be noted that the presence of nutrients (i.e. in the CEWAF+N treatment) did not yield higher concentrations of EPS, even though this treatment led to the formation of large and more abundant MOS (Chapter II and this study). These findings show that only in treatments where the chemical dispersant was applied had EPS concentrations significantly increased, and it is in only these treatments where MOS and MDS formed.



**Figure 3.3.** Average EPS concentrations in the different treatments at time points T1 (1 week), T2 (2 weeks), T3 (3 weeks) and T4 (4 weeks). Standard deviations are calculated from triplicate roller bottles for each respective treatment.

During an oil spill at sea, bacteria are the major responders, whereas eukaryotic phytoplankton are often susceptible and detrimentally affected by the toxic effects of oil hydrocarbons, as reported for the DWH spill (Parsons *et al.*, 2015). Hence, the dispersant-mediated enhancement in EPS production could largely, if not entirely, be attributed to EPS-producing bacteria that also comprise the community associated with MOS and MDS (discussed below). The dispersant may offer a labile source of carbon to these types of bacteria, which they may metabolize for growth but also convert to EPS.



**Figure 3.4.** Repeated measures ANOVA plot showing the differences in EPS concentration between the different treatments and timelines. Error bars denote the 95% confidence interval of the groups.

Various lines of speculation could be offered to explain why EPS production is induced in the presence of chemical dispersants, one of which is as a response to stressors (Wotton, 2004a,b), such as exposure to the dispersant itself and in the presence/absence of crude oil (Passow *et al.*, 2012). In this respect, the crude oil and the chemical dispersant in combination (CEWAF or CEWAF+N treatments), or the dispersant alone (SW+D), act

as the stressor and yielded heightened levels of EPS. However, crude oil alone cannot be defined as a stressor in this respect because EPS concentrations in the WAF treatment did not significantly increase compared to the untreated controls (Figures 3.3 and 3.4). Another speculation could be that a higher C/ N ratio could be produced in the presence of dispersant leading to a higher EPS production.

### **3.5.3. EPS response to dispersant and crude oil**

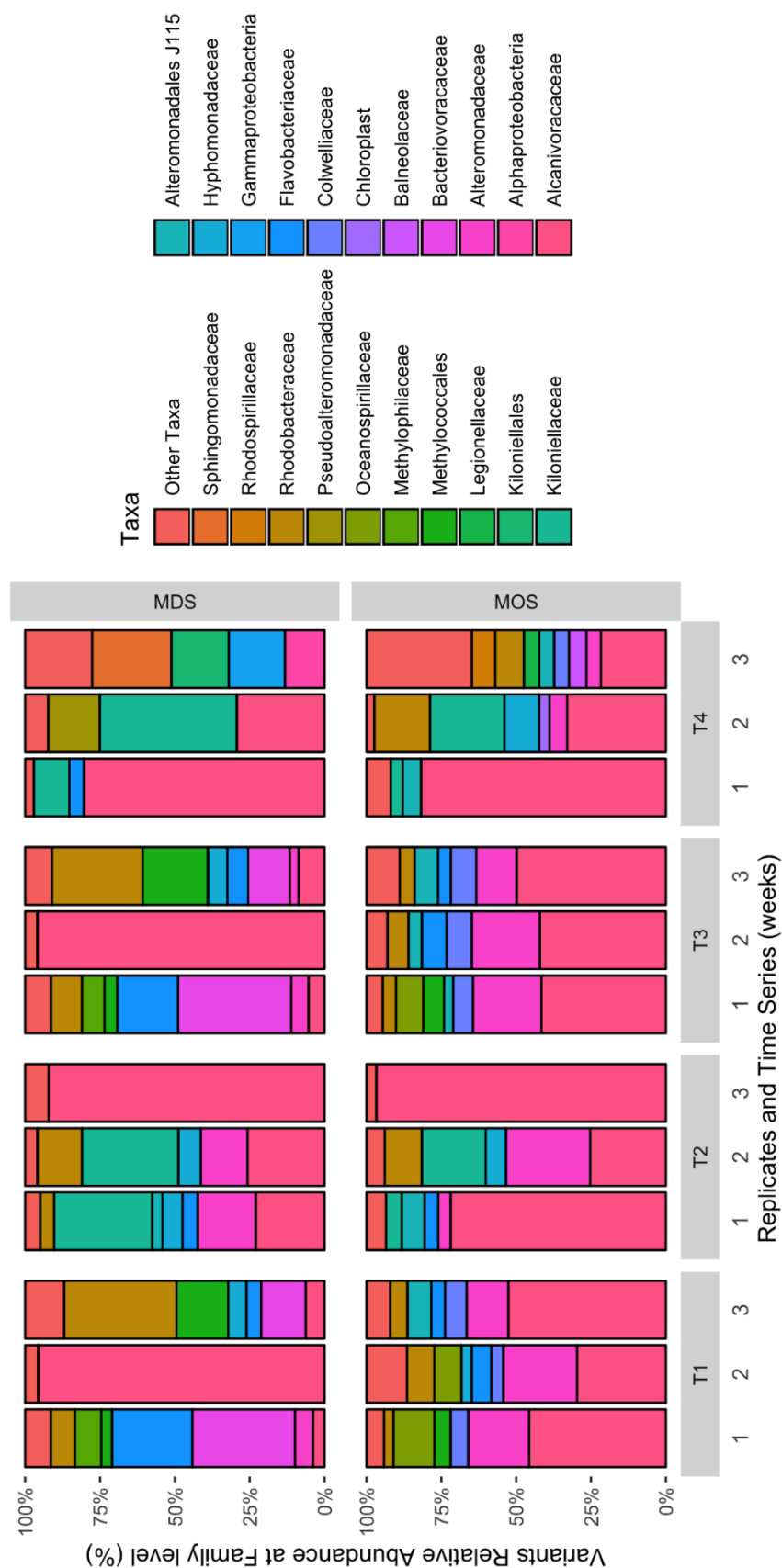
Significantly higher concentrations of EPS (PERMANOVA,  $p < 0.05$ ) were detected in treatments amended with the chemical dispersant (SW+D, CEWAF, CEWAF+N) over time compared to treatments with no added dispersant (SW, SW+N, WAF) (Figure 3.3). Based on APS analysis, average EPS concentrations across all treatments at the start of these experiments ( $T_0$ ) were  $2.7 \pm 0.7 \mu\text{g X eq. mL}^{-1}$ , which is concomitant with values reported in surface seawater of the subarctic and Arctic (Hong *et al.*, 1997; Ramaiah *et al.*, 2001), and other sites such as the Gulf of Mexico (Thornton *et al.*, 2007). Within one week ( $T_1$ ), EPS concentrations increased by ca. 10-fold (to  $33.0 \pm 8.0 \mu\text{g X eq. mL}^{-1}$ ) in the SW+D treatment, and by ca. 4-fold (to  $13.0 \pm 5.0 \mu\text{g X eq. mL}^{-1}$ ) in the CEWAF and CEWAF+N treatments. Average concentrations of EPS in these treatments remained high over the remaining duration (weeks 2 to 4) of these experiments, at 33-45  $\mu\text{g X eq. mL}^{-1}$  in the SW+D treatment, and 15-20  $\mu\text{g X eq. mL}^{-1}$  in the CEWAF and CEWAF+N treatments. Preliminary tests to evaluate whether the chemical dispersant Superdispersant-25 could be stained with Alcian Blue showed that this to be negative. In contrast, no significant change in EPS concentrations ( $p > 0.05$ ) were detected in the SW, SW+N and WAF treatments (with no added dispersant) over the entire duration of these experiments, with concentrations averaging 3.0-5.5  $\mu\text{g X eq. mL}^{-1}$ ; the exception was the WAF treatment at week 4, but the increased EPS concentration measured here can be attributed to an outlier of the triplicate samples analyzed, as shown by the large error bar range.

### **3.5.4. Bacterial community composition of MOS and MDS**

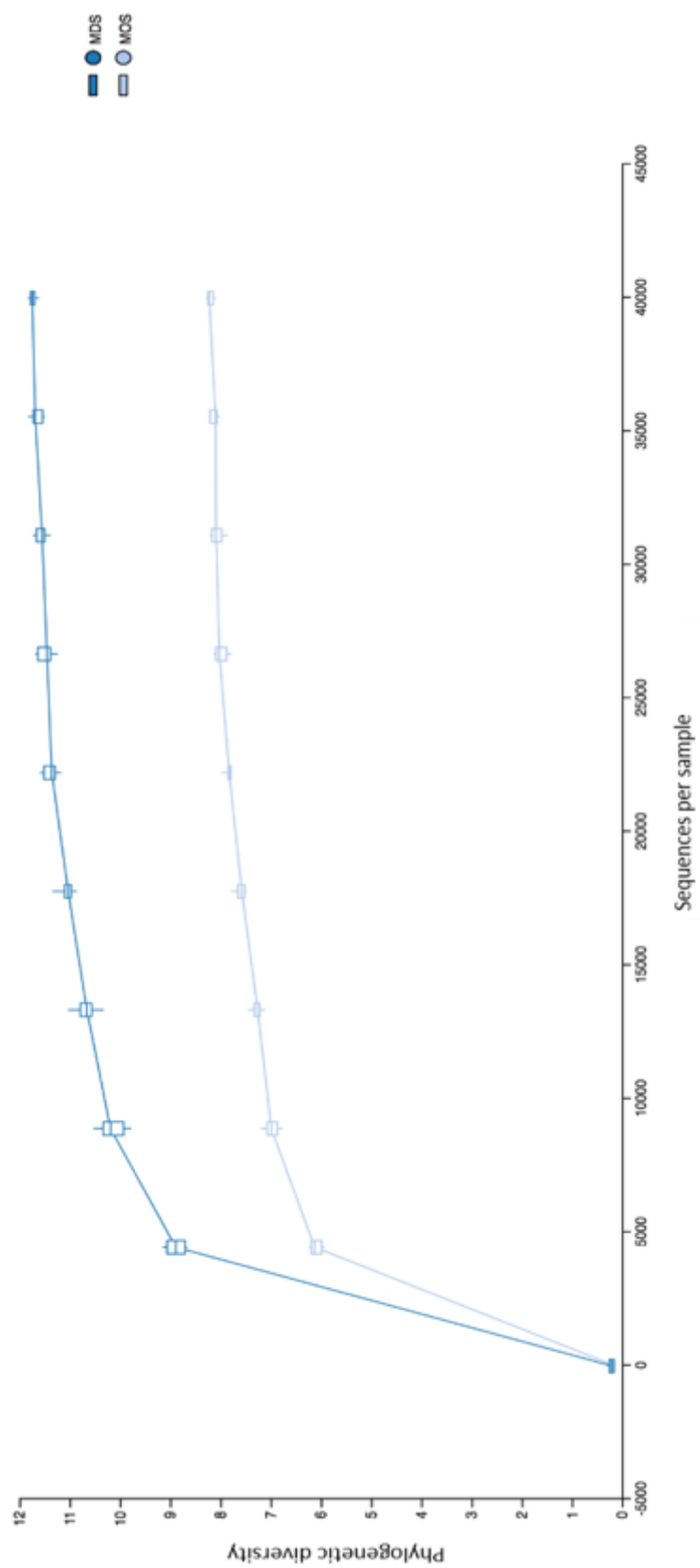
Barcoded 16S rRNA Illumina MiSeq technology was employed to study the bacterial community associated with MOS and, for the first time, with MDS over the duration of these experiments. Triplicates of MOS and MDS aggregates were sampled at each time point ( $T_1$ ,  $T_2$ ,  $T_3$ ,  $T_4$ ), and demonstrated the bacterial composition at family-level classification (Figure 3.5). No significant difference in the bacterial community composition between the MOS and MDS aggregates was identified (PERMANOVA,  $F_{24}$

= 1.12,  $p = 0.265$ ), suggesting that the oil and dispersant, which were solely associated with MOS from the CEWAF or CEWAF+N treatments, had no significant influence in structuring the community of these aggregates. However, an analysis of the similarity between these two types of aggregates at each time point revealed that MDS aggregates constitute a somewhat more diverse bacterial community than the community associated with MOS, although the difference in bacterial community composition between MDS and MOS aggregates was not significant ( $p = 0.215$ ) (Figure 3.5). Using rarefaction curves to examine the alpha-diversity of the two aggregate types indicates that MDS aggregates have a more diverse community overall than the MOS aggregate communities (Figure 3.6). Both MOS and MDS aggregates were dominated by members of the family *Alcanivoraceae*, *Alteromonadaceae* and *Rhodobacteraceae*, with percentage compositions ranging from 10% to as high as 95% of the total bacterial community composition associated with these aggregates. These taxa appear to be dominant on MOS aggregates across seasons of the year, including for members of the *Pseudoalteromonadaceae*, on MOS aggregates formed in roller bottles with seawater collected from the FSC during the winter of 2015 (Chapter II of this thesis).

It is noted that even between replicates of MOS or MDS aggregates that were sampled from the same treatment, the community composition for some showed variability. For example, two of the three replicates of MDS aggregates derived from the SW+D treatment at week 2 were identified with *Kiloniellaceae* as a major group; similarly, this was a dominant group associated with other MDS and MOS aggregates, but not with all the replicates from the same treatment. Other major groups identified, but that were not consistently identified in respective replicates from the same treatment, were *Kiloniellales*, *Flavobacteriaceae* and *Methylococcales*. This inconsistency between replicates is attributed as an indication that not all aggregates (for MDS or MOS) have the same community composition, even for aggregates sampled from different aggregates from the same bottle from the same roller bottle treatment. This microbial community patchiness has also been described for seawater at the microscale level (Azam, 1998; Fuhrman, 2009) and here we show it to occur on MDS and MOS aggregates. Based on the rarefaction curves and 3D-NMDS plots (Figures 3.6 and 3.7), the phylogenetic diversity (Faith, 1992) of the MDS aggregates appeared greater than that of the MOS aggregates.

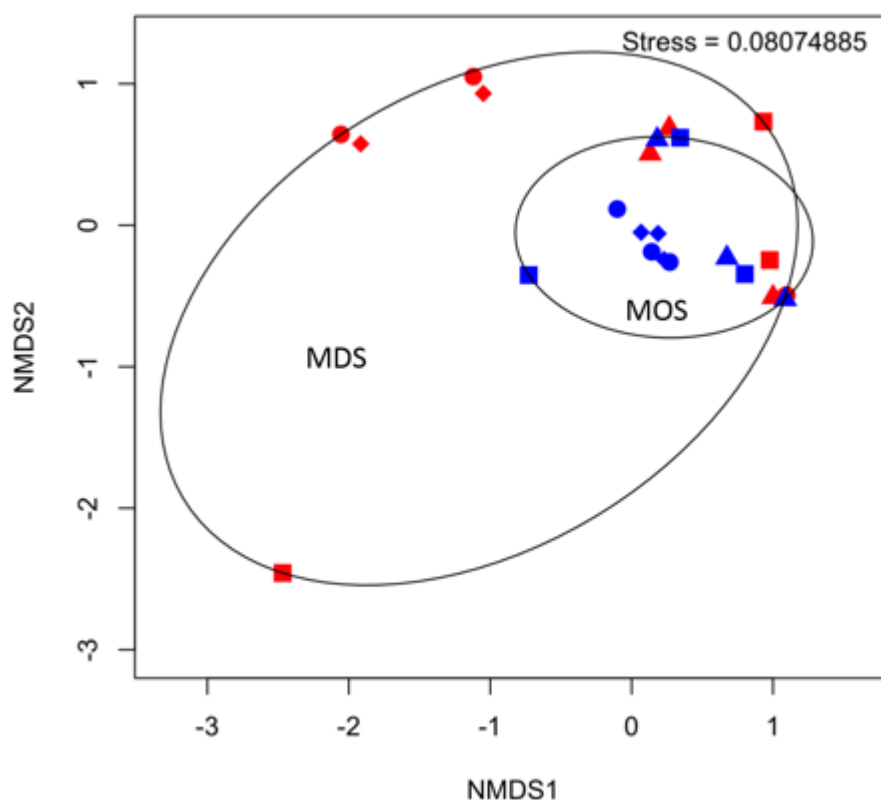


**Figure 3.5.** Composition of bacterial 16S rRNA gene MiSeq reads for MDS and MOS aggregates at weeks 1 (T<sub>1</sub>), 2 (T<sub>2</sub>), 3 (T<sub>3</sub>) and 4 (T<sub>4</sub>) from treatments SW +D and CEWAF+N, respectively. Sequences were classified to family-level taxonomy when possible and otherwise at higher level classification is shown.



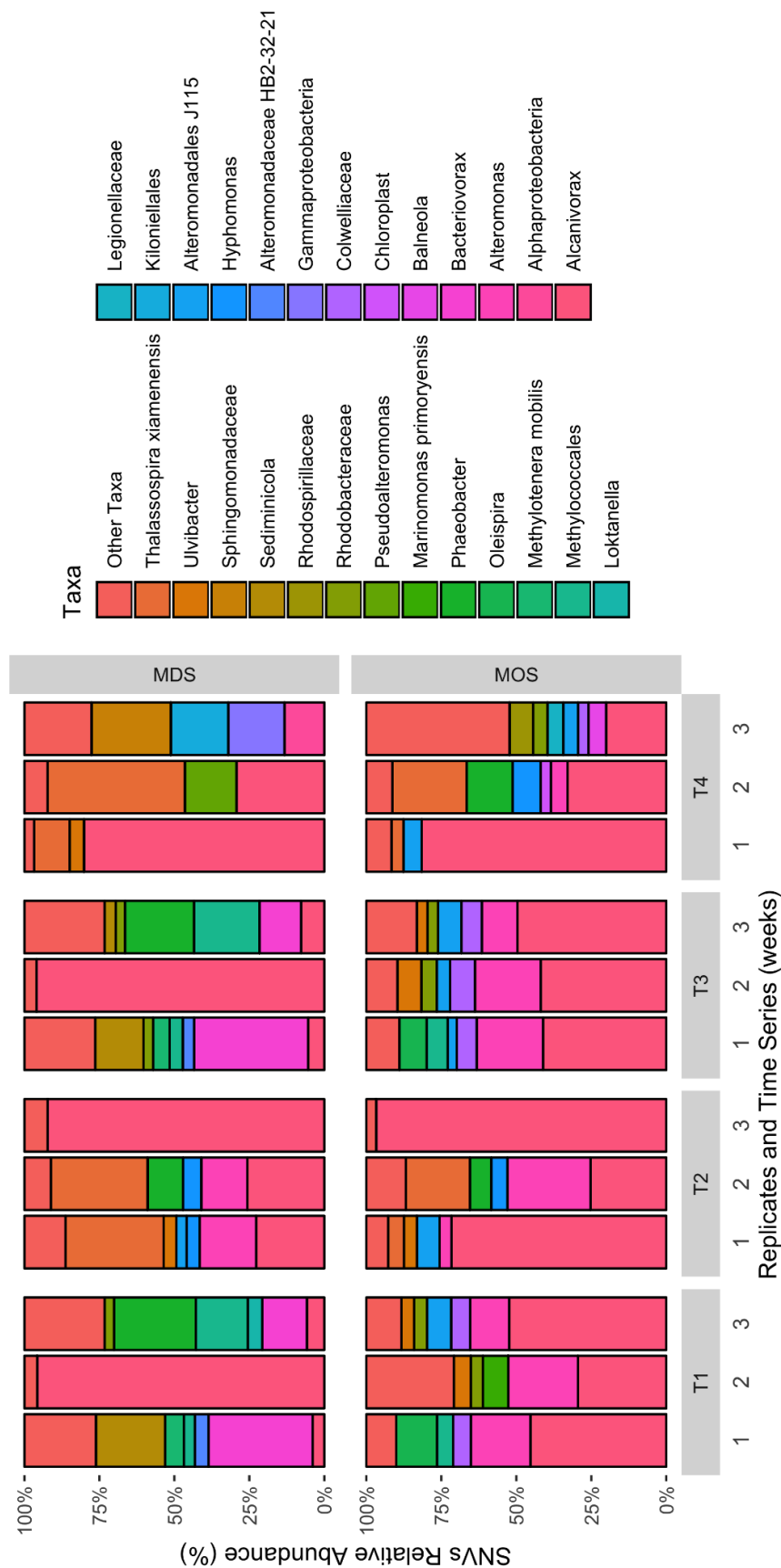
**Figure 3.6.** Rarefaction curves of observed taxa between MOS and MDS aggregates.





**Figure 3.7.** Non-metric multidimensional scaling (NMDS) of bacterial communities associated with aggregates of MDS (red symbols) and MOS (blue symbols) at week 1 (T<sub>1</sub>; circles), week 2 (T<sub>2</sub>; triangles), week 3 (T<sub>3</sub>; diamonds) and week 4 (T<sub>4</sub>; squares).

This also shows that the sequencing depth employed was sufficient to adequately characterize the bacterial diversity, as both treatment types reached an asymptote by a sequencing depth of 10,000. However, this did not result in a statistically significant difference in the alpha-diversity between the two treatments ( $H'$ ), at either the family level (ANOVA,  $F_{22}=0.486$ ,  $p = 0.493$ ) or SNVs (Single Nucleotide Variance; ANOVA,  $F_{22}=0.335$ ,  $p = 0.569$ ). The bacterial communities of both the MOS and MDS aggregates were, hence, not significantly different, as both MDS and MOS aggregates harbour a similar bacterial community, dominated by members of the family *Alcanivoraceae*. Further classification down to the genus level revealed they were dominated by members of the genus *Alcanivorax*, with minor representation by *Oleispira* (Figure 3.8) – organisms which are commonly found enriched at oil-contaminated sites and recognized in playing an important role in the biodegradation of oil hydrocarbons in marine environments (Head *et al.*, 2006; Yakimov *et al.*, 2007). This fact seems to be good evidence that dispersants contain alkanes.



**Figure 3.8.** Single nucleotide variant (SNV) relative abundance classification of bacterial 16S rRNA gene MiSeq reads for MDS and MOS aggregates at week 1 (T<sub>1</sub>), 2 (T<sub>2</sub>), 3 (T<sub>3</sub>) and 4 (T<sub>4</sub>) from treatments SW+D and CEWAF+N, respectively. Sequences were classified to genus-level taxonomy when possible and otherwise at higher level classification is shown.

Generalist hydrocarbonoclastic organisms that were also found associated with the MDS and MOS, in total, 20 aggregates studied here, included *Pseudoalteromonas* and *Alteromonas*, and whilst these organisms would be expected to contribute a role in the biodegradation of the oil associated with, or immediate vicinity of, the aggregates, these organisms are also commonly associated with producing EPS (Arias *et al.*, 2003; Mancuso Nichols *et al.*, 2004; Bhaskar and Bhosle, 2005; Gutierrez *et al.*, 2007, 2008, 2009, 2013). As such, it is possible to posit that these organisms resulted in heightened production of EPS that was observed under conditions that yielded MDS and MOS (SW+D, CEWAF, CEWAF+N). It is further posited that, since the bacterial communities associated with these two types of aggregates were similar, these communities may comprise taxa, such as *Colwellia*, with the capability to utilize the dispersant as a carbon source. Members of this genus were found as a major group in some of the MDS and MOS aggregates from the SW+D and CEWAF+N treatments respectively. Supporting this, Kleindienst *et al.* (2015a) showed potential dispersant-degrading *Colwellia* selected for in only roller bottle experiments amended with the chemical dispersant Corexit, which also bloomed during the DWH oil spill in the Gulf of Mexico.

### **3.6. Conclusion**

As documented for the DWH, Ixtoc-I and Tsesis oil spills, in the event of a spill at sea the formation of MOS is an important process leading to the transportation of the oil from the upper water column to the seafloor. This study shows that EPS concentrations in seawater become significantly higher as a response to when a chemical dispersant is applied, and interestingly this occurred irrespective of whether crude oil is present or not. Whilst we observed this for surface seawaters of the subarctic northeast Atlantic, future work should assess whether this dispersant-induced response in EPS production would also occur in other ocean regions. This response is likely conferred by EPS-producing bacteria, and while it has only been described in this and one other study, it is posited that it is a key modality in the formation of MOS when chemical dispersants are used. This study shows that in the event of an oil spill in the FSC, the use of dispersants would likely lead to the formation of MOS and MDS, and that both these types of aggregates harbor a similar bacterial community dominated by hydrocarbon degrading and EPS producing bacteria. Although the formation of MDS in the absence of crude oil has been observed in laboratory-based experiments, its significance during the application of chemical dispersants in the event of a spill at sea should not be discounted, and future investigations in this respect should consider MDS formation, including its fate and impacts.



## **Chapter IV: Effect of two different chemical dispersants on the biodegradation and bacterial response to crude oil**

#### **4.1. Overview**

This chapter investigated the potential effect of two different types of chemical dispersants on the biodegradation and bacterial community response to crude oil in surface waters of the FSC, as well as to the biodegradation of the oil. The two different dispersants evaluated were Slickgone NS and Slickgone EW as these are globally approved and stockpiled for use in the event of a major spill at sea. The experiments were performed at two different seasons of the year. It should be noted that some of the analyses were processed differently across experiments, and reasons for this will be provided in this chapter where relevant. The results reported in this chapter are in preparation for submission to a peer-review journal.

#### **4.2. Hypothesis**

In this study, two hypotheses are proposed, as follows: 1) Seasonality has an effect on the response of the bacterial community to amendment with crude oil and/or dispersant; 2) The application of different dispersants would effect a different response by the bacterial community and also on the bio-degradation of the oil.

#### **4.3. Introduction**

The use of chemical dispersants and the consequences of these on the marine environment is exemplified by the Deepwater Horizon disaster that occurred in the Gulf of Mexico in April 2010 (Hamdan and Fulmer, 2011; Kujawinski *et al.*, 2011). Approximately 760 million litres of crude oil were spilled during the disaster, during which oil-spill response authorities released approximately 2.1 million gallons of the chemical dispersant Corexit-9500A on sea surface oil slicks, including the dispersant applied directly at the leaking wellhead located 1.5 km below the sea surface. A number of studies have reported on the effect of applying dispersants on the bacterial community response and the biodegradation of the oil (e.g. Kleindienst *et al.*, 2015a,b; Chapter II of this thesis). On one hand, using dispersants can enhance biodegradation due to an increase in the surface area to volume ratio of the oil droplets in seawater (Brakstad, 2008). On the other hand, some studies have reported dispersants can suppress the activities of hydrocarbon-degrading microorganisms due to the cumulative toxicity of the oil and the dispersant together (Zahed *et al.*, 2010; Hamdan and Fulmer, 2011; Kleindienst *et al.*, 2015b). Some studies (e.g. Kleindienst *et al.*, 2015b; Chapter II of this thesis), have also shown that the use of chemical dispersants can enhance, or indeed trigger, the formation of MOS (see Chapters II and III of this thesis). However, other studies, such as Passow

*et al.* (2017), showed that dispersants suppress MOS formation by diatoms. As mentioned in Chapter I, dispersants are mainly composed of molecules belonging to emulsifiers and surfactants (Kujawinski *et al.*, 2011) and will act to reduce the surface tension between the oil and the water, by breaking up the hydrocarbon particles and increasing the surface of the oil exposed to marine organisms (Ramachandran *et al.*, 2006; Greco *et al.*, 2006). The fact that microbial-mediated aggregation and degradation processes affect the dynamics of oil at the sea surface raises questions about effects of dispersant application on microbial activities and microbial-catalysed degradation of oil (Kleindienst *et al.*, 2015b; Arnosti *et al.*, 2016; Chapter II of this thesis).

In this study, the effect of two different UK approved dispersants was examined to determine their effect on: 1) the microbial communities present in the FSC at both autumn and winter seasons; 2) the formation of MOS; 3) the formation of MDS, as reported in Chapter III; and 4) the biodegradation of the oil. This study is of great interest to be able to evaluate the planning and use of dispersants during future responses to oil spills. It is important to be able to ensure the most efficient and rapid response in order to mitigate an oil spill, thereby causing the smallest damage possible. It is relevant to consider a difference in the efficiency and effect on the ecosystem of different dispersants to be able to use the most adequate one to ensure an optimal assessment and contingency planning.

#### **4.4. Material and methods**

##### **4.4.1. Sampling area and procedures**

The samples were collected following the same methods described in Chapters II and III of this thesis (section 2.4.1 and 3.4.1 respectively). Surface seawater (20 L) was sampled from 5 m depth in the FSC, on December 15th 2015 and October 10<sup>th</sup> 2017 at the same site (FIM6a station) and same depth (60°38.12N 4°54.03W). It was collected in a 20L carboy at two different time points during a MRV *Scotia* cruise on December 2015 and October 2017. The seawater was stored in a plastic carboy at 4°C on board for one week and then transported to Heriot-Watt University (Edinburgh, UK) for downstream analysis. Data for conductivity was collected from the boat, temperature and depth (CTD) for both cruises are presented in Table 4.1.

**Table 4.1.** CTD parameters of FIM06a during the two different seasons

Parameters	Dec-15	Oct-17
Depth (m)	5	5
Pressure (Pa)	50000	50000
Temperature (°C)	8.74	10.89
Salinity (‰)	35.259	35.053
Chlorophyll a (mg/m <sup>3</sup> )	0.06	0.47

In addition, satellite images from the MODIS Aqua data of NASA (sea surface on 8-day averages) as produced with the Giovanni online data system – developed and maintained by the NASA GES DISC were collected. Satellite images for this during the sampling times are presented in the results section.

#### **4.4.2. Laboratory Experiments: Microcosm set up**

The experimental setup used simulated surface sea conditions of the FSC during each season (specified below), and kept the incubations in constant rolling motion. The same oil used in previous experiments, Schiehallion crude oil, was used here (provided by BP). The two dispersants used were diluted with seawater before use and applied to the natural seawater at a dilution of 1:10 (dispersant/oil) in all treatments. These dilutions resulted in dispersant concentrations that are currently recommended to the oil and gas industry by the UK government (<https://www.gov.uk/government/publications/marine-pollution-contingency-plan> MMO, government UK). Slickgone EW (S-EW) and Slickgone NS (S-NS) were provided by DASIC International LTD.

Table 4.2 summarises the composition of each treatment. As can be seen, no water accommodated fractions were made for this experiment and direct application of oil and dispersant was used respecting the dilutions of each recommended by industry and government. This was in order to represent more realistically the ecosystem in the case of an oil spill, where the oil slick would stay on the surface for a certain amount of time. A total of sixty-three Pyrex© glass tubes (20 mL) were set up on test tube racks and installed in a shaker (VWR® Incubating Orbital Shaker, Model 3500I) at an angle of around 45° with gentle rotation (50 rpm). Each of the 9 treatments was done in triplicates. At each time point, two replicates of each treatment were sacrificed for the crude oil analysis to analyse for biodegradation or to cover any potential loss of a tube during the experiment. Treatments were analysed at different time points: first day of the experiment (T<sub>0</sub>), 2 weeks (T<sub>2</sub>), 4 weeks (T<sub>4</sub>), and 6 weeks (T<sub>6</sub>), at which point the experiment was



stopped.

**Table 4.2.** Details of the components in each treatment

Treatment	Components
SW	Seawater
SW+N	Seawater+ nutrients
SW+S-EW+N	Seawater+ Slickgone EW+ nutrients
SW+S-NS+N	Seawater+ Slickgone NS+ nutrients
SW+O+N	Seawater+ oil+ nutrients
SW+O+S-EW	Seawater+ Slickgone EW+ oil
SW+O+S-NS	Seawater+ Slickgone NS+ oil
SW+O+S-EW+N	Seawater+ Slickgone EW +oil+ nutrients
SW+O+S-NS+N	Seawater+ Slickgone NS +oil+ nutrients

**Note:** SW: seawater; N: nutrients; S-EW: Slickgone EW; S-NS: Slickgone NS; O: oil.

Water samples were aliquoted in 10 mL triplicates (3x 20 mL tubes). Firstly, an entire volume of 2.4 L of seawater was dispensed into clean and autoclaved 20 mL plexiglass tubes with teflon-lined caps. For each treatment, 3 tubes were filled up with 10 mL of sea water from the FSC. Microcosms were established and maintained at 8°C for December 2015 and 10°C for October 2017 on the shaker device in the dark. The treatments with oil were enriched with 500µL filter-sterilized crude Schiehallion oil; dispersant treatments were enriched with 50 µL of dispersant, Slickgone EW (S-EW) or Slickgone NS (S-NS). The treatments SW+O +N+ S-EW and SW+O+N+S-NS were enriched with both crude oil (500 µL) and one of the dispersants (50 µL). The published chemical characteristics of these two dispersants are presented (Tables 4.3 and 4.4). Since the exact chemical composition of these dispersants is proprietary, the information presented here should be indicative only.

**Table 4.3.** Chemical properties of Slickgone NS (DASIC international LTD).

Slickgone NS	Concentration
Anionic surfactant	1-10% w/w
Odourless kerosene	>50% w/w

**Table 4.4.** Chemical properties of Slickgone EW (DASIC international LTD).

Slickgone EW	Concentration
Kerosene-odourless-distillates (petroleum)	40-50%
Sodium dioctylsulphosuccinate	1 - 10%
Monomethyl ethers of dipropylene glycol	1 - 10%

At the designated time points – start of the experiment ( $T_0$ ), 2 weeks ( $T_2$ ), 4 weeks ( $T_4$ ) and 6 weeks ( $T_6$ ) – samples were collected for 16S rRNA gene amplicon comparison analyses and MOS formation analysis. At each time point, 7mL of seawater (under the oil/ dispersant slick) of each tube was collected for DNA analysis being careful to not sample aggregates. Also, at the same time point, records of any visually observed oil attribute changes, MOS formation observations were made. Some aggregates were sampled and transferred with several droplets of deionised water prior to their examination under the light microscope. Only the light and the contrast of the picture taken were modified with Image J software (Rueden *et al.*, 2017). For the experiment set up in October 2017, at each time point two tubes for each treatment were sacrificed and processed immediately for hydrocarbon analysis, as described in section 4.4.5.

#### **4.4.3. Genomic DNA extraction**

For both seasons, genomic DNA was extracted as described in Chapter II – i.e. for each treatment at each time point for this 6-week experiment. Briefly, 7mL samples were filtered using a glass column filtration system (Millipore) with 45 mm polycarbonate membrane filters (0.22  $\mu\text{m}$  pore size; Isopore) and the filters were subsequently stored at  $-20^\circ\text{C}$ . For extraction, the membrane filters were placed in liquid nitrogen and then ground to a powder. The liquid nitrogen was permitted to evaporate from each tube and the remaining content (powdered filter membranes) was extracted according to the method of Tillet and Neilan (2000). Purified DNA was stored at  $-20^\circ\text{C}$  for subsequent molecular analysis.

#### **4.4.4. Barcoded amplicon sequencing and analysis**

Barcoded amplicon 16S rRNA gene sequencing and analysis was conducted in two different ways for each season. A description of this differential approach for each of the two experiments (i.e. different for each season) is split into two sections, as follows.

##### *4.4.4.1. Bacterial community composition analysis for the December 2015 experiment*

Barcoded 16S rRNA gene MiSeq sequencing, targeting the V3-V4 hypervariable region, was employed to analyse the bacterial community present in the nucleic acid extractions from each treatment over the 6-weeks duration of the experiments at time points  $T_0$ ,  $T_1$ ,  $T_2$ ,  $T_4$  and  $T_6$ , as described in Chapter II. In summary, the primers used

were 341f (CCTACGGGNGGCWGCAG) (Muyzer *et al.*, 1993) and 806r (GGACTACHVGGGTWTCTAAT) (Caporaso *et al.*, 2011, 2012). Both primers also had Illumina MiSeq overhangs attached to their 5' ends. Barcodes were not added at this point of the PCR. Following receipt of purified PCR amplicons at the sequencing facility, a second stage PCR was conducted for the addition of the golay barcodes. These barcodes were unique to each treatment to enable multiplexing.

All samples were sequenced using the Illumina MiSeq platform (Illumina 2 x 250 V.2 kit) at the University of Liverpool Centre for Genomic Research ([www.liverpool.ac.uk/genomic-research](http://www.liverpool.ac.uk/genomic-research)); sequences were demultiplexed prior to receipt at our laboratory. Subsequent processing was conducted using the MiSeq SOP (accessed: Sept 2016) cited within the MOTHUR program (Kozich *et al.*, 2013). In brief, contiguous sequences were constructed from paired end sample reads and examined using MOTHUR (v1.36.1) formatted RDP database (v.14). Operational taxonomic units (OTUs) were clustered based on 97% sequence identity and subsampled to 35,000 sequences per sample to eliminate sampling bias during subsequent diversity examination.

#### *4.4.4.2. Bacterial community composition analysis for the October 2017 experiment*

In October, the method for this analysis was modified to improve the quality of the data to sequence. In this case, barcoded 16S rRNA gene MiSeq sequencing, targeting the V4 hypervariable region, was employed to analyze the bacterial community of the different treatments over the 6-weeks duration of the experiments at time points T<sub>0</sub>, T<sub>1</sub>, T<sub>2</sub>, T<sub>4</sub> and T<sub>6</sub>. PCR of the 16S rRNA gene was performed in duplicate 25 µl reactions, which were subsequently pooled to increase PCR product yield. Each reaction comprised 10.5 µL molecular biology grade water, 12.5 µL of Platinum Hot Start Master Mix, 0.5 µL each of 10 µM forward (515f) and reverse (806r) primers, and 1 µL of template DNA. The primers used were 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACNV GGGTWTCTAAT) (Caporaso *et al.*, 2011, 2012). Both primers had Illumina MiSeq overhangs and unique golay barcodes added to the 5' ends. All PCR products were purified by GFX PCR purification (#GE28-9034-70, Sigma, UK). All samples were sequenced via the Illumina MiSeq platform (Illumina 2 x 250 V.2 kit) at the Edinburgh Genomics sequencing facility (Edinburgh University, UK); sequences were demultiplexed prior to receipt. Subsequent processing was conducted using the MiSeq SOP (accessed: Sept 2016) cited within the MOTHUR program (Kozich *et al.*, 2013). In brief, contiguous sequences were constructed from paired end sample reads and

examined using MOTHR (v1.36.1) formatted RDP database (v.14). Operational taxonomic units (OTUs) were clustered based on 97% sequence identity and subsampled to 35,000 sequences per sample to eliminate sampling bias during subsequent diversity examination.

#### **4.4.5. Hydrocarbon extraction and analysis for the October 2017 experiment**

This analysis was conducted in the Lyell Centre with the help of Thomas Wagner's research group that specifically designed this protocol for this study. A small fraction of volatile short-chain components was lost during the rotator evaporation step although the preservation was good enough to conserve from C<sub>10</sub> to C<sub>37</sub>. This result gives us enough information to analyse the biodegradation patterns in our experiment. Hydrocarbon analysis was performed for the experiment comprising the October 2017 season. It was not performed for the December 2015 samples. This was due to me not having access to any laboratory in 2015 equipped with a GC-MS capable for doing the hydrocarbon analyses on these samples. To be able to analyse the degree of hydrocarbon biodegradation that occurred in the oil-amended treatments, controls had been set up in parallel. For this, a sample of only pure Schiellalion crude oil, as well as a NSO1 reference oil (oil of known composition by the research group), were processed in an identical manner, including the extraction from seawater, as the rest of the sub-samples (see below) - these represented the controls (or reference) for comparison to the treatments.

Duplicates of each treatment tube were sacrificed at T<sub>2</sub>, T<sub>4</sub> and T<sub>6</sub> for extraction of total petroleum hydrocarbons (TPH) by placing the contents of the tube into 250 mL separatory funnels with dichloromethane (DCM) at treatment mix (see treatment components on Table 4.2) to DCM ratio 1:2 (check ratio). The DCM fraction was removed and the treatment mix re-extracted an additional three times. Crude oil was serially extracted with DCM. The supernatant containing the hydrocarbons was removed from the separated oil-water mixture using a glass pipette. Residual oil was dissolved in petroleum ether and added to the supernatant oil. The oil sample was then diluted with DCM to ca. 5 mL and dried by the addition of a small amount of anhydrous sodium sulphate. Each time the oil was processed, it was weighed before and after extraction. These gravimetric data were used to calculate the original sample weight and the weight of oil remaining.

A known aliquot of the sample (from which we want to extract the hydrocarbons)

corresponding to 30 mg was transferred to a 10 mL vial. An aliquot of the reference oil (Schiellalion oil) was weighed directly into a vial and diluted with ca. 0.3 mL DCM. Positive controls (squalane and 1,1-binaphthyl) were added as surrogate standards at 0.5% and 0.05% by weight of the oil (w/w), respectively. Samples were analysed in triplicate and a procedural blank including the standards was also prepared daily. A small amount of aluminium oxide was added to all the vials containing the samples and DCM was removed under a gentle stream of gaseous nitrogen; the aluminium oxide was stirred during evaporation of the solvent to ensure an even distribution of the hydrocarbon sample. Hydrocarbons were extracted in two phases: aromatics and aliphatics, using a chromatographic column with silica and topped with aluminium oxide. From the bottom to the top, the first 60% of the column was filled with silica, then the sample mixed with aluminium oxide and finally, over it, a layer of aluminium oxide was laid. To extract the first fraction of hydrocarbons (aliphatic hydrocarbons), 50 mL of petroleum ether was added into the column and the resulting fraction was collected into a beaker. To extract the second fraction of hydrocarbons (aromatic hydrocarbons), 70 mL of petroleum ether: DCM (2:5 v/v) was added into the column and then collected with the corresponding fraction. The two fractions containing the aliphatic and aromatic hydrocarbons from each sample were purged with N<sub>2</sub> until dry and then collected in vials.

Subsequently, 500 µL of hexane was added in each vial and vortexed to ensure resuspension of the hydrocarbons in the hexane. The samples were stored at -20 °C until analysed on a Hewlett Packard 5890 GC fitted with a split injector flame ionisation detector (FID) (310 °C) and an HP-5 capillary column (J&W, 30 m x 0.25 µm film thickness, GCMS). Samples were injected using a Hewlett Packard 6890 injector. The oven program was 50 °C (2min) - 5 °C (52min) - 300 °C (20 min) giving a total time of 74 min. Chromatographic data were acquired and processed using a Chromaleon Chromatographic Data System (Thermo Scientific). The aliphatic content of the samples was calculated using the manually integrated area under the whole GC-MS chromatogram, drawing a linear baseline from the start of the solvent to the end of the acquisition. Corresponding total area for the procedural blank (which also contained the surrogate standards) was then subtracted from the total area obtained for the samples. Ratios of *n*-alkanes to acyclic isoprenoid hydrocarbons (pristane/nC17 and phytane/nC18) were used as convenient indicators of oil degradation (Peters *et al.*, 2005). A similar procedure was done for the aromatic hydrocarbons using a few other ratios indicative of biodegradation – e.g. 2-methylnaphthalene/1-methylnaphthalene

(2MN/1MN) (Larter *et al.*, 2012); phenanthrene/9-methylphenanthrene (P/9MP); 3,1,2-methylphenanthrene/9,1,1-methylphenanthrene (3MP/9MP) (Bennett and Larter, 2008); 3-methylphenanthrene/2-methylphenanthrene (3MP/2MP) (Bennett *et al.*, 2013). Results of *n*-alkane hydrocarbon profiles are here plotted in relation to their peak area in counts per minute (cpm). Those are the number of ions that hit the detector per unit of time. Now there is a correlation between the numbers and the concentration of the compound.

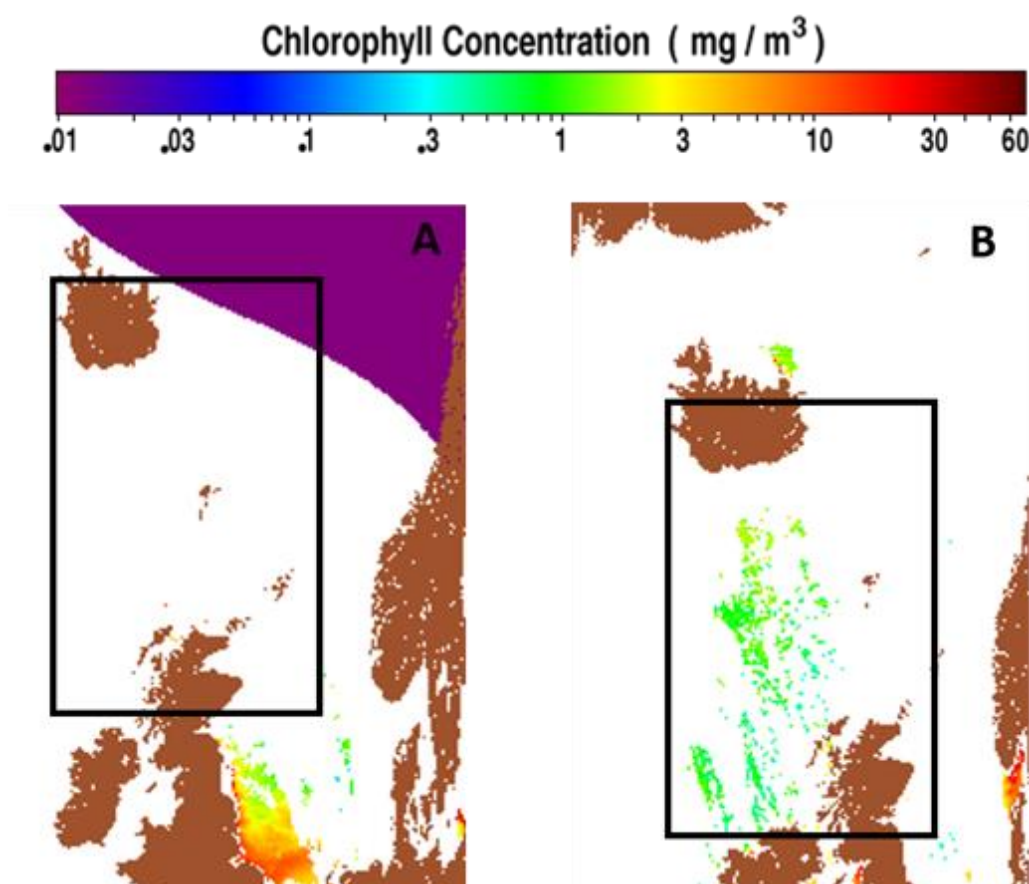
#### **4.4.6. Statistical Analyses**

Statistical analyses were done for each of the two experiments (i.e. for each season) independently due to the fact that the methods in the bacterial community composition analyses were different although the results will be compared in the Discussion. For each season, differences between treatments are evaluated using PERMANOVA analysis and the relative abundances of sequences obtained using MiSeq were compared using an NMDS plot to visualize  $\beta$ -diversities of each sample for both treatment and time point. Moreover, Shannon-Weiner diversity ( $H'$ ) was measured (base log10) and compared using an analysis of variance to determine significant differences between diversity of treatments and time points sampled. All data was log transformed to meet the assumptions of parametric analysis. For hydrocarbon ratios, differences were analysed using repeated ANOVA analyses.

### **4.5. Results**

#### **4.5.1. Environmental Parameters for Both Seasons**

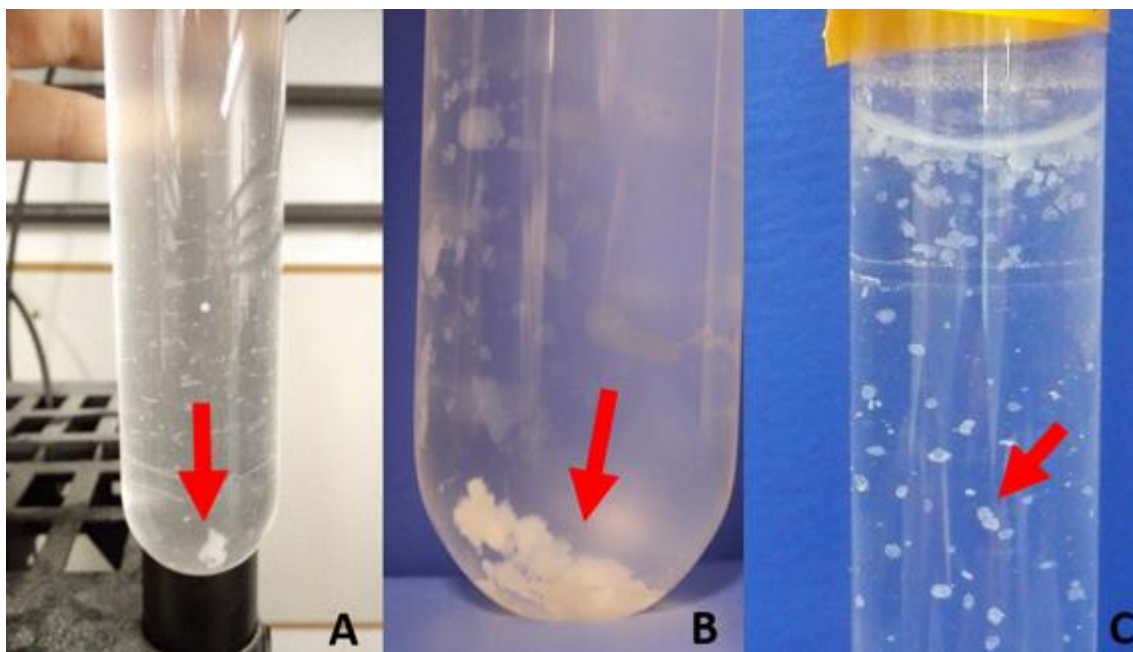
Chlorophyll *a* concentrations were higher in autumn 2017 (0.47 mg/m<sup>3</sup>) than in the winter of 2015 (0.06 mg/m<sup>3</sup>) following the data from the CTD (Table 4.1). However, this shows different values in satellite images collected by NASA (Figures 4.1A and 4.1B). This could be due to a bad quality or an artefact in the satellite images. Also, the temperature recorded by the CDT cast was around 2°C higher in October 2017 (10.79 °C) than in December 2015 (8.64°C). Salinity seems similar at both seasons (approx. 35.1 ‰) in both cases.



**Figure 4.1.** Location of the sampling area chl *a* concentrations (in mg/m<sup>3</sup>) in surface waters of the FSC at the respective sampling times A: December 2015; B: October 2017. These were available online from the MODIS Aqua data of NASA (sea surface on 8-day averages) as produced with the Giovanni online data system – developed and maintained by the NASA GES DISC.

#### 4.5.2. MOS and MDS Formation in Both Seasons

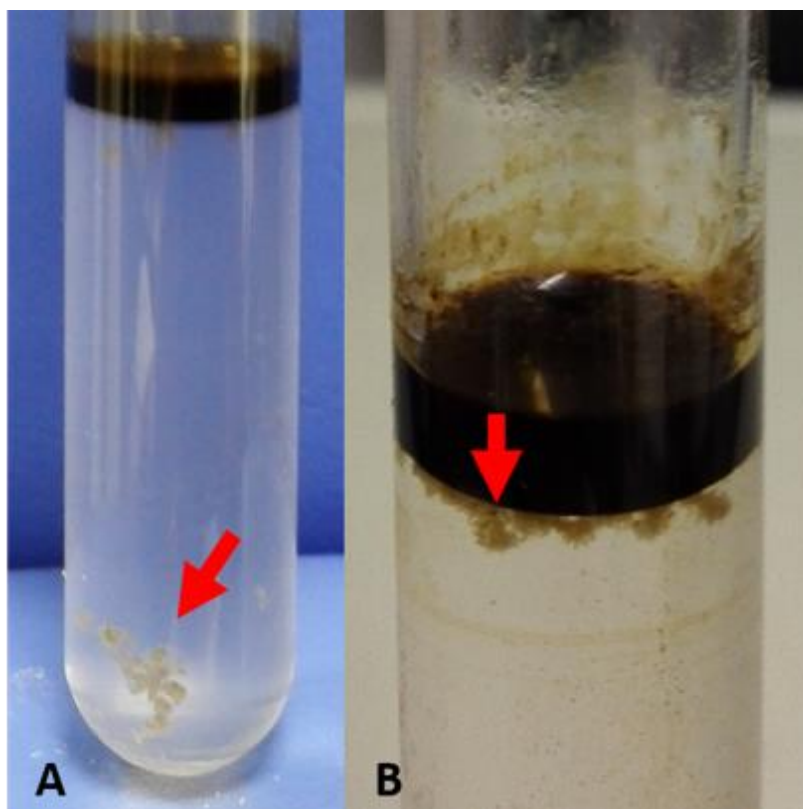
During both experiments, some macroscopic flocculation formed in these microcosms that resembled the aggregates observed in the previous experiments (Chapters II and III of this thesis). MOS and MDS formation occurred in the same way and was the same time for both seasons. In the microcosms, the most rapid oil snow formation was observed in SW+O+N+SW-EW/S-NS treatments within 5 days of incubation, and two days later (at 7 days) in the SW+O+S-EW/S-NS treatment. Roundish, fractal-looking aggregates were visible and increased substantially in size, number and shape. During the two first weeks, some aggregates were observed to merge, forming larger aggregates. After T<sub>2</sub>, average aggregate sizes appeared to remain unchanged for the rest of the experiment. SW+O+N treatments did not reveal visible marine oil snow. However, MDS was observed in the SW+N+S-EW and S-NS treatments.



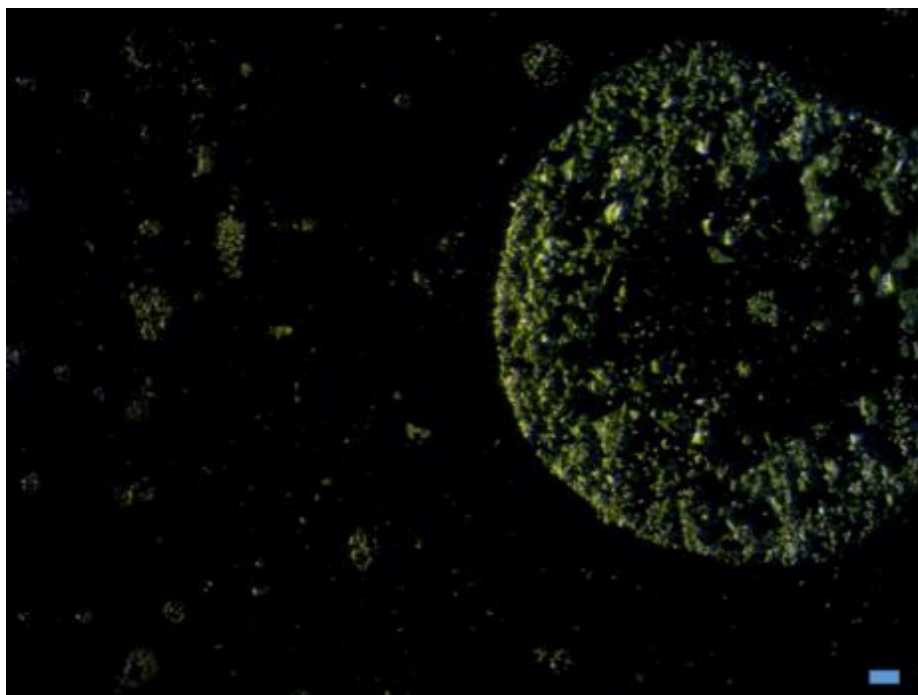
**Figure 4.2.** (A) MDS formation in treatments amended with Superdispersant 25 in the absence of crude oil (Chapter II of this thesis) compared to (B) MDS formation in treatments with Slickgone NS and with (C) Slickgone EW, again without crude oil.

MDS aggregates were abundant for both seasons and with both types of dispersant tested here, but their appearance and texture was somewhat distinct to that observed for MDS that formed with Superdispersant 25 (Chapter II of this thesis) which were ‘fluffy’ and larger in size (see Figure 4.2). These aggregates that formed in treatments with Superdispersant 25 (Figure 4.2A) did show some similarity to those that formed with Slickgone NS (Figure 4.2B) - they were quite gluey when sampled, showing a high content of a viscous substance. MDS that formed with Slickgone EW, however, appeared in shape similar to fish scales not especially mucoid, drier than the MDS aggregates formed with other dispersants although quite sticky (Figure 4.2C). MOS also formed in both SW+O+S-EW/S-NS and SW+O+N+S-EW/S-NS treatments for both seasons. Figure 4.3 shows the MOS aggregates observed at the end of the experiment with each respective dispersant. The MOS aggregates showed a similar morphology independent of the kind of dispersant used and when the seawater was collected - hence, only two images of these MOS aggregates are presented. As shown in Figure 4.3, the aggregates appear brownish and fluffy, and contained oil droplets entrained within. As stated previously, seasonality did not seem to influence the time point at which MOS and MDS formed, nor their appearance and textural qualities.





**Figure 4.3.** MOS aggregates that formed in the SW+O+N+S-EW/S-NS treatment with the dispersant (A) Slickgone EW and (B) Slickgone NS.



**Figure 4.4.** An unstained MOS aggregate that formed in SW+O+N+S-EW, as observed under brightfield light microscopy (x40). Bar, 10 $\mu$ m.

Figure 4.4 shows a MOS aggregate (right half of the image) that formed in treatment SW+O+N+S-EW when observed under the light microscope. The aggregate has a roundish shape, although smaller aggregate fragments are observed (left half of the image), possibly the result of the decomposition of larger aggregates during their handling or in natural conditions, due to the dynamics of the seawater. No stain was used to observe this aggregate, only the aggregate and the light microscopy were used here. The grey/brownish colouration of the aggregate may be due to reflectance produced by the light hitting on oil and/or dispersant droplets present on the aggregate surface.

#### **4.5.3. Bacterial community from December 2015 and its response to oil and dispersant**

The bacterial community composition in December 2015 across the different treatments and its response to oil and/or dispersant (or combination of both), is presented in Figure 4.5. In the SW treatment, the bacterial community remained quite stable during the first two first weeks since commencing these experiments. By week 4, an increase in members of the family *Rhodobacteraceae* and decrease in the rest of the families was observed. The bacterial community of SW at T<sub>6</sub> is quite similar in terms of families present to the one at T<sub>0</sub> although the proportions of each are different. For instance, the family *Rhodobacteraceae* present in low proportions (20%) at T<sub>0</sub> had a higher relative abundance at T<sub>6</sub> (45- 70%).

In the SW+N treatment, the bacterial community at T<sub>0</sub> is quite different to the one of the SW treatment. The bacterial community composition remained relatively unchanged until the end of the experiment and reminiscent of that in the SW treatment in the first two weeks. The taxon *Flavobacteriaceae*, absent at the SW treatment, appears at T<sub>2</sub> of the SW+N treatment. Moreover, in SW+N, the taxon *Rhodobacteraceae* is present in a very low percentage (10-15%) and almost disappears in the SW+N treatment across the time compared to T<sub>6</sub> in SW treatment. Finally, the proportion of *Gammaproteobacteria* seems to increase in SW+N to 25-30% compared to SW where it represents a smaller relative abundance of 15-20% of the bacterial community composition. It looks like the nutrients are a factor influencing the bacterial community composition.

In the treatment of SW+O+N, there is an important proportion of *Alcanivoraceae* (50-75%) representing the bacterial community for the duration of the experiment.

*Alteromonadaceae* (10%), *Oceanospirillaceae* (5%) and *Flavobacteriaceae* (5%) are present but in smaller proportions. *Oceanospirillaceae* and *Alteromonadaceae* that are present during the first 4 weeks, seem to disappear at T<sub>6</sub>. At T<sub>6</sub>, the bacterial community is basically represented by *Alcanivoraceae* (80%), *Flavobacteriaceae* (10%), and Other taxa (10%). There seems to be a decrease in diversity over time where there is a reduction in the number of representative taxa, from three at T<sub>6</sub> to an average five at T<sub>2</sub>.

In the treatments SW+N+S-EW and SW+N+S-NS, the community composition profile seems quite different depending on the dispersant used. In the case of the dispersant S-EW (Slickgone EW), the bacterial community seems quite similar over time, mainly represented by *Alcanivoraceae* (45%), 25-30% of *Pseudoalteromonadaceae* (25-30%), and *Vibrionaceae* (25-30%), followed by a similar proportion of other taxa. At T<sub>6</sub>, a small proportion of *Rhodospirillaceae* appears for the first time (~5%). In the case of the other dispersant, S-NS (Slickgone NS), the bacterial community composition seems more diverse. During the two first weeks, the taxon *Vibrionaceae* (45-50%) seems quite dominant followed by *Gammaproteobacteria* (10-20%), *Alteromonadaceae* (10-15%) and Other taxa (5-10%). However, from T<sub>4</sub>, the bacterial community composition changes quite drastically and the proportion of *Vibrionaceae* decreases to 5% while the taxon *Pseudoalteromonadaceae* (40%) appears to be pretty dominant followed also by the appearance of the *Alcanivoraceae* (~30%) that comes to replace *Gammaproteobacteria* and *Alteromonadaceae*. Finally, at T<sub>6</sub>, the proportion of *Pseudoalteromonadaceae* decreases to 10%, the taxon *Gammaproteobacteria* appears again (10%) and *Rhodospirillaceae* (15-20%) appears for the first time. The appearance of *Rhodospirillaceae* at T<sub>6</sub> also occurred using the other dispersant (S-EW).

For both the SW+O+S-EW and SW+O+S-NS treatments with oil present and one or other dispersant, the bacterial community composition profile was quite similar over the duration of the experiment. In both cases, these treatments seem to have a higher diversity of taxa compared to previous treatments. In general, under this treatment with S-EW or S-NS, the bacterial community profiles show quite predominant families such as *Alteromonadaceae*, *Colwelliaceae*, *Flavobacteriaceae*, *Oceanospirillales\_incertae*, *Pseudoalteromonadaceae* and *Rhodobacteraceae* seem to be all in the same proportions (15%-20%).

In the case of SW+O+S-EW (with Slickgone EW), there is a similar proportion of the taxa as mentioned previously, although it is possible to observe an increase of *Flavobacteriaceae* in T<sub>6</sub> compared to T<sub>2</sub> and T<sub>4</sub> as well as the disappearance of the taxa *Colwelliaceae* and *Oceanospirillales\_incertae* at T<sub>6</sub> which were present (10-15%) at T<sub>2</sub> and T<sub>4</sub>. In the case of SW+O+S-NS, the taxon *Flavobacteriaceae* increases from T<sub>2</sub> (~10%) to T<sub>6</sub> (~45%) and *Oceanospirillales\_incertae* present at T<sub>2</sub> and T<sub>4</sub> disappears at T<sub>6</sub>. However, the addition of nutrients to the same treatment seems to have an effect on the bacterial community composition of the SW+O+N+S-EW and SW+O+N+S-NS treatments compared to the results without nutrients of SW+O+S-EW and SW+O+S-NS described above. In this case, the bacterial community profiles are different depending on the dispersant used (Slickgone EW or Slickgone NS).

In the treatment with S-EW, the bacterial community is mainly represented by members of the *Vibrionaceae* (50%) at T<sub>2</sub> increasing to the end of these experiments at T<sub>6</sub> (80%). This proportion of *Vibrionaceae* is followed by a small presence of *Halomonadaceae* (10%) at T<sub>2</sub> and T<sub>6</sub> although it disappears at T<sub>4</sub>; and *Alteromonadaceae* (25%) at T<sub>2</sub> and 10% at T<sub>4</sub>, disappears at T<sub>6</sub>. A marked relative abundance of members of the *Vibrionaceae* were present in treatments containing nutrients and dispersants (S-EW or S-NS), whereas their presence was barely or not detected in the other treatments where dispersant was absent; the high percentage relative abundance of *Vibrionaceae* in the SW+O+S-EW treatment for time point T<sub>6</sub>, however, may be an anomaly as its replicate (T<sub>6A</sub>) shows quite a distinct profile with no presence of *Vibrionaceae*. All across the timeline, Other taxa represent around 15% of the bacterial community.

The SW+O+N+S-NS treatment shows quite a different profile with clearly greater diversity, although sharing the presence of the *Vibrionaceae* (10-15%) with the treatment with S-EW. During the two first weeks, *Alcanivoraceae* (15%), *Alteromonadaceae* (10%), *Gammaproteobacteria* (20%), *Oceanospirillales\_incertae* (~45%), *Vibrionaceae* (10-30%) and Other taxa (around 20%). This seems to change at T<sub>4</sub> where the proportions of *Vibrionaceae* decreased to 10-15% of relative abundance and the taxa *Alcanivoraceae* and *Alteromonadaceae* seem to increase in relative abundance (30-35% and 20-25% respectively) compared to T<sub>2</sub>. Moreover, new members of the bacterial community appear at T<sub>4</sub> such as *Gammaproteobacteria* (5-10%), *Halomonadaceae* (~5%) and *Oceanospirillaceae* (5%).

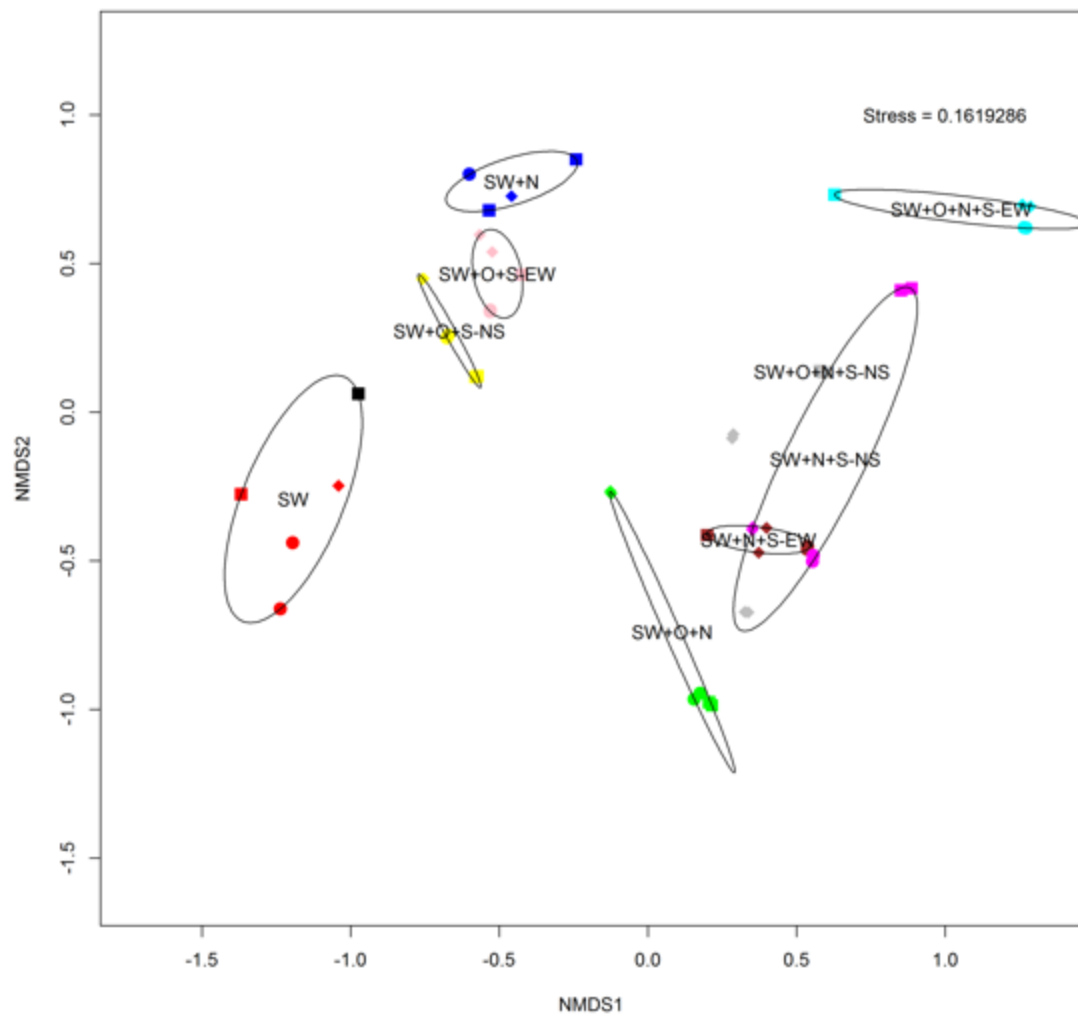


**Figure 4.5.** Bacterial community composition at family-level classification for each of the different treatments over 6 weeks incubation with seawater collected from the FSC in the winter (December) of 2015. SW, seawater; SW+N, seawater with nutrients; SW+N+S-EW, seawater with Slickgone EW and nutrients; SW+N+S-EW+NS, seawater with Slickgone NS and nutrients; SW+N+S-EW+NS+Oil, seawater with Slickgone EW and oil; SW+N+S-EW+NS+Oil+Nutrients, seawater with Slickgone NS and oil; SW+N+S-EW+NS+Oil+Nutrients+Oil, seawater with Slickgone NS, oil and nutrients; SW+N+S-EW+NS+Oil+Nutrients+Oil+Nutrients, seawater with Slickgone NS, oil and nutrients. Taxa contributing more than 5% are shown. A and B represent replicates.

The members of the *Alcanivoraceae* (50%) were predominant in this treatment with S-NS over the course of the incubation, whereas these organisms were largely undetectable in the same treatment with S-EW. Other families present in the bacterial community composition profile with the S-NS were *Alteromonadaceae*, *Halomonadaceae*, *Oceanospirillaceae* and *Flavobacteriaceae*.

Based on the bacterial community composition analysis across treatments, the SW+O+N+S-NS has a more similar bacterial community composition to that of the SW+O+N treatment (without dispersant) than the SW+O+N+S-EW treatment. A PERMANOVA analysis indicates a significant difference in bacterial community composition between treatments (ANOSIM,  $R=0.695$ ,  $p=0.001<0.05$ ) and between time points (ANOSIM,  $R=0.066$ ,  $p=0.001<0.05$ ), but not between replicates (ANOSIM,  $R=0.002$ ,  $p=0.859>0.05$ ) as would be expected for the latter. This is exemplified in the NMDS plot shown in Figure 4.6 which represents the similarity in bacterial community composition of the different treatments and different time points over the course of these experiments. This NMDS shows that the bacterial community composition of the different treatments is quite well defined. It shows how similar the bacterial community composition is for each respective treatment over 6 weeks. To take one example, the community of the SW+O+N treatment is markedly different to that of the SW+N and SW+O+N+S-EW treatments.

However, the bacterial composition of the SW+N+S-EW and SW+N+S-NS treatments are quite similar, and interestingly similar also to the SW+O+N+S-NS treatment. Results show that there is no significant difference (Kruskal-Wallis,  $p$  value= $0.4726>0.05$ ) in diversity across time nor treatments. Table 1 (Appendix C) summarises all Shannon Wiener values corresponding to each treatment at each time point.



**Figure 4.6.** Non-metric multidimensional scaling (NMDS) plot showing the similarity of the bacterial community (in seawater from December 2015) for each treatment at the different time points sampled over the 6-week duration of the experiment. The stress achieved is indicated in the top right of the plot. Each time point is represented by a different symbol: T<sub>0</sub> (black square); T<sub>2</sub> (square); T<sub>4</sub> (diamond); T<sub>6</sub> (closed circle). Treatments are represented by colours: SW (red), SW+N (dark blue), SW+N+S-EW (brown), SW+N+S-NS (magenta), SW+O+N (green), SW+O+S-EW (pink), SW+O+S-NS (yellow), SW+O+N+S-EW (cyan), and SW+O+N+S-NS (grey). Ellipses shown surrounding symbols represent grouping of the treatment types with 95% confidence interval.

#### 4.5.4. Bacterial Community from October 2017 and its Response to Oil and Dispersant

The bacterial community composition for October 2017 across treatments and time points is represented in Figure 4.7. In a general overview, it is possible to see that the bacterial community seems quite homogenous across the treatments and the time series. It is possible to see an enrichment of oil-degrading bacteria such as *Alteromonadaceae*, *Alcanivoraceae* and putative EPS-producing bacteria such as members of the families *Rhodobacteraceae* and *Rhodospirillaceae*.

For the SW treatment, the bacterial community composition at T<sub>0</sub> seems to be dominated by *Alcanivoraceae* (50%) followed by *Alteromonadaceae* (20%), Other taxa (15%) and *Colwelliaceae* (5%). The bacterial community does not seem to differ on T<sub>2</sub> although a couple of new members appear such as *Flavobacteriaceae* and *Rhodobacteraceae* both present with 5% of relative abundance. Finally, at T<sub>6</sub>, the proportions of taxa to the previous weeks seem similar only showing a small increase of the representation of *Flavobacteriaceae* (~20%) and *Rhodobacteraceae* (10-15%) in the bacterial community. In the case of the SW+N, the bacterial community composition seems to slightly differ from T<sub>0</sub>. Although having at T<sub>2</sub> a high representation of *Alcanivoraceae* (30-75%) compared to T<sub>0</sub>, the families *Oceanospirillaceae* (~ 25%) and *Rhodobacteraceae* (~15%) appear when absent in SW at T<sub>0</sub> and at the same time point. Moreover, members of the family *Alteromonadaceae* seem to have disappeared compared to the SW treatment. At T<sub>4</sub>, the bacterial community composition shows a similar profile to the previous weeks although *Alteromonadaceae* seems to have disappeared and some other new taxa appear such as *Piscirickettsiaceae* and *Vibrionaceae*. Finally, at T<sub>6</sub> *Hyphomonadaceae* appears in one of the replicates (~ 20%) although the rest of the representative taxa stay almost the same. The bacterial community composition seems generally homogenous across treatments with a general high abundance of *Alcanivoraceae* (more than 50%) that decreased with time in the enriched treatments with oil and/or dispersant.





Other families present in smaller proportions are *Rhodobacteraceae* (5-10%), *Rhodospirillaceae* (5-10%). Only the treatment SW+O+N+S-NS seems to show a higher diversity and seems to be different from the rest of treatments in terms of bacterial community composition. This treatment shows a high proportion of *Piscirickettsiaceae* (~ 50%) in weeks T<sub>4</sub> and T<sub>6</sub> and a smaller proportion of *Alcanivoraceae* compared to the rest of the treatments.

In the treatments SW+N+S-EW and SW+N+S-NS, the profiles of the bacterial community seem to share the same taxa but in different proportions. In the case of SW+N+S-EW at T<sub>2</sub>, the bacterial community seems basically composed of *Alcanivoraceae* (15-30%), *Alteromonadaceae* (20-25%), Other taxa (around 25%). In one of the replicates of T<sub>2</sub>, *Rhodobacteraceae* shows a relative abundance of around 60%. However, the other replicate does not show the presence of this taxa. The next four week samples T<sub>4</sub> and T<sub>6</sub> seem quite similar in bacterial community composition with a dominance of *Alcanivoraceae* (50%), followed by *Alteromonadaceae* (20-25%) and small representation of the taxa *Vibrionaceae*, *Rhodobacteraceae* and *Oceanospirillaceae* (all 5-10%). The group Other taxa keeps representing around 20-25% of the bacterial community composition under this treatment.

In the SW+N+S-NS treatment, the replicates do not seem very similar so it is difficult to describe the bacterial community composition profiles. At T<sub>2</sub>, there is a dominance of *Alcanivoraceae* (wide range between 30-75%) followed by the taxa *Flavobacteriaceae* (10%), *Pseudoalteromonadaceae* (20%), *Rhodobacteraceae* (10-50%) and other taxa (5-10%). It is not very different in taxa representation than in the case of S-EW, however the taxa *Alteromonadaceae* is not present in this case. At T<sub>4</sub>, only one replicate is represented and the bacterial community is dominated by *Rhodobacteraceae* (~50%), *Flavobacteriaceae* (15%), *Oceanospirillaceae* (25%) and other taxa (10%). At T<sub>6</sub>, a reduction of diversity occurs leading to a few taxa representing the bacterial community composition: *Alcanivoraceae* (25- 50%), *Alteromonadaceae* (around a 60%, only in one replicate), *Vibrionaceae* (10%), *Pseudoalteromonadaceae* (10%) and Other taxa (10-25%).

In the treatment SW+O+N, the same taxa than in the previous treatments seem to be present although there is a clear dominance of *Alcanivoraceae* (~ 50-80%) from T<sub>2</sub> to T<sub>6</sub>. This profile, however, is not that different from T<sub>0</sub> and the SW treatment. At T<sub>2</sub>,

after the strong dominance of *Alcanivoracaceae* (40-60%) followed by *Rhodospirillaceae* (20-25%) and *Alteromonadaceae/ Oceanospirillaceae* and Other taxa (all 5-20%). At T<sub>4</sub>, the dominance of *Alcanivoracaceae* becomes stronger and reaches between 70-90% of relative abundance of the bacterial community followed by around 20% of *Vibrionaceae* and 5-10% of Other taxa. At this time point, *Rhodobacteraceae* seems to disappear as well as *Oceanospirillaceae*. Finally, at T<sub>6</sub>, the same bacterial community composition than at T<sub>4</sub> is observed although *Oceanospirillaceae* (5%) and *Pseudoalteromonadaceae* (10%) appear at this stage.

The treatments SW+O+S-EW or S-NS show a similar composition in bacterial community compared to the rest of the treatments, characterised again by a dominance of *Alcanivoracaceae*. The use of the two different dispersants does not seem to change the bacterial community composition too much. In the case of SW+O+S-EW at T<sub>2</sub>, except for one of the replicates that shows a predominance of *Piscirickettsiaceae* (50%) followed by *Rhodospirillaceae* (10%) and *Zhongshania* (30%), all the rest of the time points show a similar structure. *Alcanivoracaceae* (75-95%) is followed at T<sub>4</sub> by *Rhodobacteraceae* (15-20%), *Rhodospirillaceae* and *Proteobacteria* (each 10-15%). At T<sub>6</sub>, *Proteobacteria* and *Rhodospirillaceae* disappear but *Rhodobacteraceae* remains (15%).

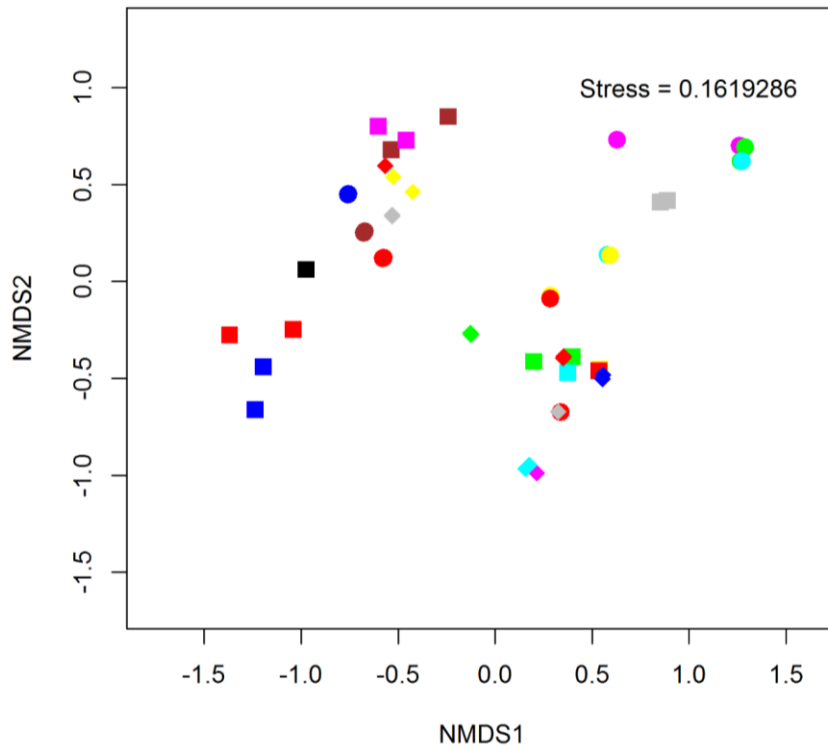
In the case of SW+O+S-NS, a similar pattern is drawn, although some taxa not appearing in the case with S-EW appear. At T<sub>2</sub>, the bacterial community is again dominated by *Alcanivoraceae* (50%), followed by a similar proportion *Alteromonadaceae*, *Colwelliaceae*, *Vibrionaceae* and Other taxa (each 10-20%). This diversity seems to decrease at T<sub>4</sub> where only a few major groups seem to represent the treatment: *Alcanivoraceae* (60-75%), *Rhodospirillaceae* (20%) and *Piscirickettsiaceae* (10%). At T<sub>6</sub>, one of the replicates shows a higher diversity while the other one does not. The one that does not is strongly dominated by *Alcanivoracaceae* (90%). However, the other replicate shows a much lower presence of *Alcanivoraceae* (~15%) and the presence of other taxa such as *Alteromonadaceae* (40%), *Flavobacteriaceae* (15%), *Hyphomonadaceae* (15%), *Rhodobacteraceae* (20%) and *Vibrionaceae* (around 15%) and Other taxa.

In the case of this same last treatment but with the addition of nutrients, SW+O+N+S-EW and SW+O+N+S-NS, the bacterial community composition seems to differ from the rest of the treatments. For example, *Rhodospirillales unclassified* seem to only appear under the application of S-NS but not S-EW.

Firstly, for the treatment SW+O+N+S-EW, there is only replicates for the time point T<sub>6</sub>. At T<sub>2</sub>, *Alcanivoracaceae* (50%) is followed by the presence of *Alteromonadaceae* (~ 25%), *Colwelliaceae* (15%), *Zhongshania* (10%) and Other taxa (~ 15%). At T<sub>4</sub>, the diversity seems to decrease, and *Alcanivoracaceae* (60%) seems the taxon most abundant and is only followed by two other taxa: *Piscirickettsiaceae* (5%) and other taxa (around 15%). Finally, at T<sub>6</sub>, the replicates seem quite different and show a wide range of abundance of *Alcanivoraceae* (25-80%) and the presence of minor taxa such as *Hyphomonadaceae* (25%), *Rhodobacteraceae* (15%), *Vibrionaceae* (15%), *Colwelliaceae* (10%) and *Rhodospirillaceae* (5%).

Secondly, in the case of SW+O+N+S-NS, there are replicates for each time point. However, those seem quite different if compared. At T<sub>2</sub>, one of the replicates shows a strong predominance of *Alcanivoracaceae* (~85%) completed by other taxa. In the other replicate, *Alcanivoracaceae* is not present but the taxa *Hyphomonadaceae* (~60%) is dominant followed by *Flavobacteriaceae* (20%), *Rhodobacteraceae* (15%) and Other taxa (20%). At T<sub>4</sub>, the taxon *Proteobacteria* appears and is dominant in one of the replicates (~50%) followed by the presence of *Rhodospirillales unclassified* (15%), *Zhongshania* (25%) and Other taxa (around 20%). However, in the other replicate, *Alcanivoracaceae* is dominant (~50%) and other taxa such as *Alteromonadaceae*, *Colwelliaceae*, *Rhodobacteraceae* (each 5-15%) followed by other taxa (25%) contribute the rest. Finally, at T<sub>6</sub>, a similar pattern to T<sub>4</sub> is observed, also in terms of replicates. However, this time new members appear to be present at T<sub>4</sub> such as *Hyphomonadaceae* (10%) and *Flavobacteriaceae* (5%).

Statistically, the bacterial community composition did not seem to change across the time points of the experiment. However, these differences between treatments (ANOSIM, R=0.159, p=0.607>0.05), time points (ANOSIM, R=0.047, p=0.690>0.05) and replicates (ANOSIM, R=0.010, p=0.797>0.05) are non-significant, as indicated by ANOSIM analyses. Based on the bacterial community composition analysis, comparing treatments, it seems that bacterial communities do not strongly differ. A PERMANOVA analysis indicates a non-significant difference in bacterial community composition between treatments (ANOSIM, R=0.178, p>0.05) and between time points (ANOSIM, R=0.695, p>0.05) in this case.



**Figure 4.8.** Non-metric multidimensional scaling (NMDS) plot showing the similarity of the bacterial community (in seawater from October 2017) for each treatment at the different time points sampled over the 6-week duration of the experiment. The stress achieved is indicated in the top right of the plot. Each time point is represented by a different symbol: T<sub>0</sub> (black square); T<sub>2</sub> (square); T<sub>4</sub> (diamond); T<sub>6</sub> (circle). Treatments are represented by colours: SW(red), SW+N (dark blue), SW+N+S-EW (brown), SW+N+S-NS (magenta), SW+O+N (green), SW+O+S-EW (pink), SW+O+S-NS (yellow), SW+O+N+S-EW (cyan) and SW+O+N+S-NS (grey). Ellipses to show grouping of the treatment types (with 95% confidence interval) are not shown because there is not any 95% similarity in bacterial community composition between treatments.

This is exemplified in the NMDS plot (Figure 4.8) which represents a perfect example of non-significant differences between samples. This plot only highlights that there are not significant differences in bacterial community composition between treatments nor time points and they all similar. Results show that there is no significant difference (Kruskal-Wallis,  $p$  value=0.4728>0.05) in Shannon Wiener diversity indices across time nor treatments. In Table 2 (Appendix C) all Shannon Wiener values corresponding to each treatment at each time line are summarised.

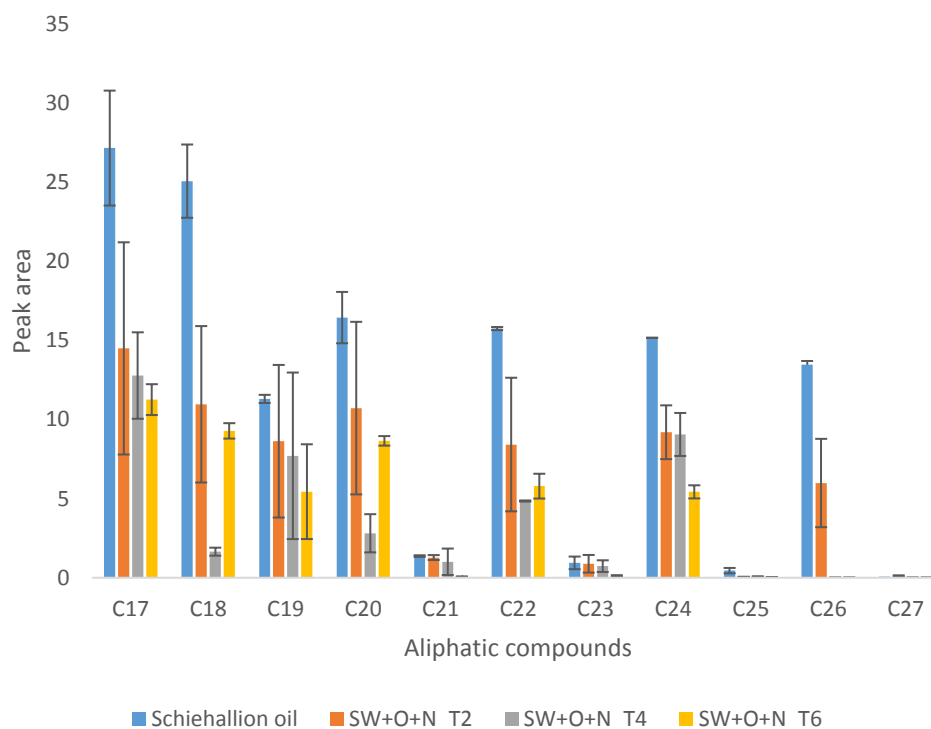
#### 4.5.5. Hydrocarbon analysis in October 2017

To determine for biodegradation of the oil, the aliphatic and aromatic fractions of the oil were analysed at 6 weeks for treatments that had been amended with oil (SW+O+N, SW+O+N+S-EW, SW+O+N+S-NS) and this was compared to the same at the commencement of these experiments. For the aliphatic fraction, *n*-alkanes (C<sub>17</sub>-C<sub>27</sub>) were analysed in oil extracts from the different treatments and compared to their presence in the oil at the commencement of these experiments at T<sub>0</sub>. Peak areas are in counts per minute that is a unit linearly proportional to the concentration of the compound (see section 4.4.7).

Firstly, in Figure 4.9, it is interesting to highlight that the peak areas of the alkanes C<sub>18</sub> and C<sub>20</sub> are higher at T<sub>6</sub> than at T<sub>4</sub>, this not following the normal pattern. Schiehallion crude oil profile on Figure 4.9 represents the crude oil *n*-alkane profile at T<sub>0</sub>. It is used as a control for Figures 4.9 and 4.10. Figure 4.9 shows the *n*-alkane profile in the SW+O+N treatment compared to that in the original oil. It is worth noting that the oil used is originally somewhat weathered judging by its *n*-alkane profile, particularly evidenced by the peak area values for the *n*-alkanes C<sub>17</sub>-C<sub>18</sub> (25-30 counts/minute), which drop to a peak area of 10-15 counts/minute for *n*-alkanes of longer chain length analysed. Nonetheless, a clear biodegradation pattern occurred in the SW+O+N treatment over time as shown by a decrease in peak area and, thus, denotes a decrease in the concentration of these *n*-alkanes over time. This is confirmed by significant differences in peak area between treatments (Repeated ANOVA,  $p=0.008<0.05$ ) and time (Repeated ANOVA,  $p=0.039<0.05$ ), but not between replicates (Repeated ANOVA,  $p=0.627>0.05$ ).

Figure 4.10 shows the profile of the C<sub>17</sub> to C<sub>27</sub> *n*-alkanes in the SW+O+N+S-EW and SW+O+N+S-NS treatments over time. With SW+O+N+S-EW, the concentrations of *n*-alkanes decreased over the 6-week duration of these experiments (denoted by the decreasing peak area of these alkanes) and is thus indicative of their biodegradation. However, in the case of the SW+O+N+S-NS treatment, there is no apparent biodegradation of the *n*-alkanes as the peak area seems to fluctuate and even increase in occasions at some time points – there is no clear pattern of biodegradation having occurred when using S-NS (Slickgone-NS). Analysing the data separately, there is already significant differences comparing the treatment and the control. In the case of S-EW, there are significant differences between treatment (Repeated ANOVA,

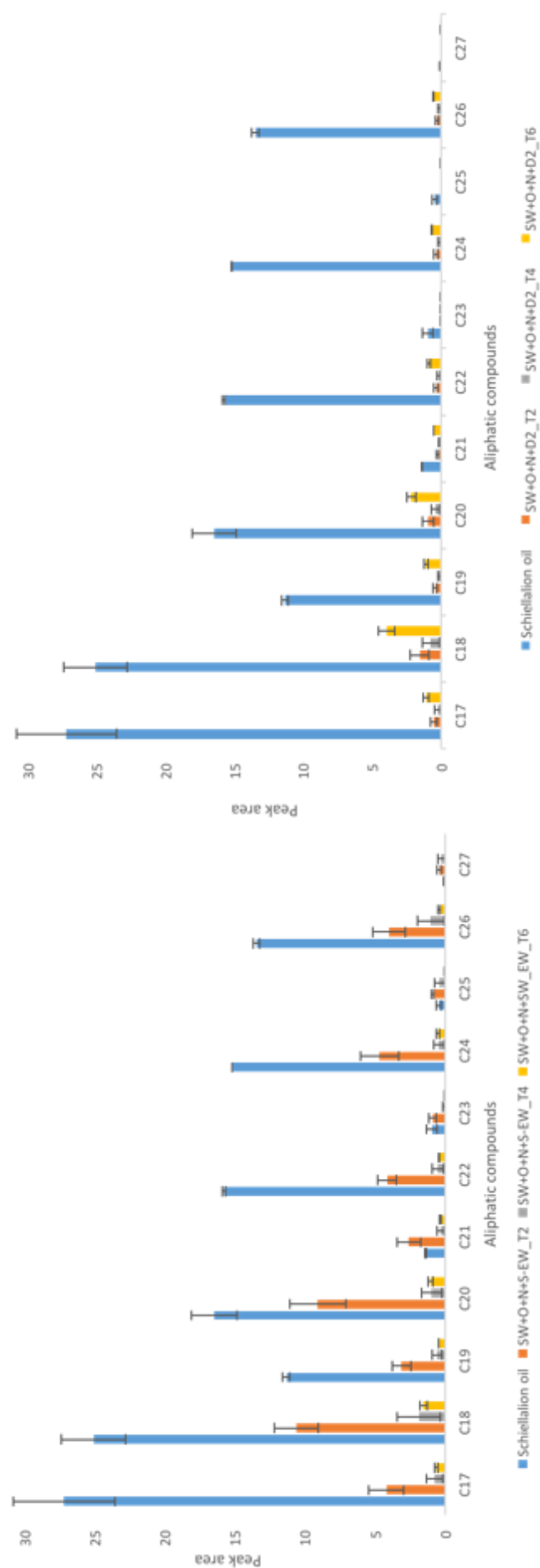
$p=0.01<0.05$ ) and time (Repeated ANOVA,  $p=0.029<0.05$ ) but not between replicates (Repeated ANOVA,  $p=0.785>0.05$ ).



**Figure 4.9.** Peak area and their respective standard deviation bars obtained by the profiles of the GC-FID of the aliphatic compounds in the Schiehallion oil ( $T_0$ ) and SW+O+N treatments at the three different time points  $T_2$  (after 2 weeks),  $T_4$  (after 4 weeks) and  $T_6$  (after 6 weeks). Peak area is in counts per minute (counts of ions that hit the detector per minute).

For the treatment with S-NS, there are significant differences between treatment (Repeated ANOVA,  $p=0.03<0.05$ ) and time (Repeated ANOVA,  $p=0.035<0.05$ ) but not between replicates (Repeated ANOVA,  $p=0.09>0.05$ ).

Comparing both treatments, there is a significant difference between the control, the treatment with S-EW and the treatment with S-NS (Repeated ANOVA,  $p=0.008<0.05$ ) as well as between time points (Repeated ANOVA,  $p=0.049<0.05$ ). However, there is no significant difference between replicates within treatments (Repeated ANOVA,  $p=0.669>0.05$ ).



**Figure 4.10.** Peak area and their respective standard deviation bars obtained by the profiles of the GC-MS of the *n*-alkanes in the SW+O+N+S-EW and SW+O+N+S-NS treatments at the three different time points T<sub>2</sub>, T<sub>4</sub> and T<sub>6</sub>. Peak area is in counts per minute (counts of ions that hit the detector per minute).



All ratios of the aromatic fraction obtained after analysis through GC/FID were calculated and analysed, but are not shown here since there were generally not significant differences between aromatic ratios Pr/C<sub>17</sub> (Repeated ANOVA, between treatments  $p=0.721>0.05$ ; between time points  $p=0.538>0.05$  nor replicates  $p=0.738>0.05$ ) and Ph/C<sub>18</sub> (Repeated ANOVA,  $p>0.05$ , between treatments  $p=0.052>0.05$ ; between time points  $p=0.172>0.05$  nor between replicates  $p=0.159>0.05$ ; see Appendix C, Tables 3 and 4). The rest of the ratios representing the aromatic fraction of the hydrocarbons did not show any significant difference either between treatments, time points nor replicates (Repeated ANOVA,  $p\text{-value}>0.05$ , see Tables 5-8 in Appendix C). This means that, generally, during the six weeks duration of the experiment, these ratios did not change significantly across treatments nor over time.

## **4.6. Discussion**

### **4.6.1. Environmental data**

During the winter months, the northeast Atlantic is generally characterised by low nutrient, phytoplankton and chlorophyll-a concentrations (a proxy for labile organic carbon, Miller *et al.*, 2015; Debes *et al.*, 2007; McQuatters-Gollop *et al.*, 2007), and this is observed in this study by a low concentration of chlorophyll-a in December 2015 collected by Niskin bottles when collecting the CTD data. However, in October (autumn), a second bloom of these constituents can occur (the first bloom reported in summer, Berx *et al.*, 2013) and this is translated into a high concentration of chlorophyll a in this study in October 2017 compared to December 2015. In winter, phytoplankton blooms are uncommon while in the autumn month blooms provide a surge of organic and inorganic nutrients into the upper layers of the water column. This differential organic carbon and particle organic matter (POC) between these seasons could explain the differential bacterial community response for both seasons in the crude oil/dispersant enrichment experiments. In winter, when POC concentrations are low, the bacterial community may consume more of the oil, and possibly also the dispersant, as major carbon sources, assuming trace nutrients are not limiting. In autumn, however, the higher levels of organic carbon (phytoplankton biomass), POC and inorganic nutrients could be preferred over as a more labile source of carbon compared with oil and/or dispersant. Indeed, a positive relationship between bacterial abundance and chlorophyll a has been described in marine ecosystems (Bird and Kalff, 1984; White *et al.*, 1991). Temperature could also be a factor influencing the bacterial community composition. This could suggest that in October

2017 (autumn), the bacterial community is likely to be abundant and diverse due to a high concentration of chlorophyll *a* and a higher temperature of the surface sea-water than in December 2015 (winter) where it could be more specialised in oil and dispersant as main source of carbon and low temperature.

Seasonality effects on marine bacterial communities have been described before as leading to differences in bacterial community composition over seasonal scales (e.g. Pinhassi *et al.*, 2006). Seasonality can have direct repercussions on nutrient availability to microbial communities, and this has a key role in defining the ocean primary productivity and thus to regulate the bacterial and phytoplankton community composition, diversity and succession (Smayda and Reynolds 2001, 2003; Pinhassi *et al.*, 2006). This is observed in this study where at the start of these experiments ( $T_0$ ), the seawater bacterial community composition is already different at both seasons, being more diverse in December 2015 than in October 2017. Moreover, it is possible to say that the bacterial community composition in this study differs quite strongly between seasons.

#### **4.6.2. MOS and MDS formation**

MOS and MDS aggregates formed in the same amount of time (within 5 days) in both seasons and resembled those observed in previous studies (Kleindienst *et al.*, 2015a; Chapters II and III of this thesis). Even in the absence of crude oil, aggregates formed in the SW+N amended with S-EW or S-NS treatments and also resembled those observed in studies by Kleindienst *et al.* (2015a,b) who used seawater from the Gulf of Mexico supplemented with the dispersant Corexit 9500A – the dispersant used in large quantities by BP on sea surface oil slicks and pumped directly at the leaky Macondo well-head during the DWH spill (National-Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011).

In this study, both types of Slickgone dispersants (EW and NS) exerted the same effect in inducing the formation of aggregates. For MDS that formed in the presence of Slickgone EW, the aggregates appeared physically different to those that formed with the dispersant Superdispersant 25 (Chapters II and III of this thesis) and with Slickgone NS (this study). In the case of Superdispersant 25 (Chapter II and III of this thesis) and Slickgone NS (this study), MDS aggregates were very large and sticky (like glue), suggesting they contained high concentrations of EPS that may have been released extracellularly by EPS-producing bacterial (c.f. Chapter III). On the other hand, MDS aggregates that formed with Slickgone EW did not appear gluey or sticky, suggesting that use of this dispersant may not stimulate EPS production by the bacterial community.

Whilst the appearance and textural qualities of MDS aggregates were different depending on the dispersant used (i.e. Slickgone EW or Slickgone NS), MOS aggregates that formed in the SW+O+N treatments amended with S-EW or S-NS were observed to have a similar appearance and textural qualities. From these results, it is important to note that different types of dispersants will lead to producing MDS and/or MOS with different physical qualities, and one could therefore posit that this could also effect a differential response by the microbial community and also affect the biodegradation of the oil – factors which were further investigated below.

These results reveal that in the event of an oil spill in the FSC during the winter and autumn, and under conditions where a dispersant is used by oils-pill response authorities, MOS would be expected to form on the sea surface. Furthermore, the application of nutrients, as is sometimes used as a bioremediation approach, would potentially amplify the abundance and size of the MOS particles – as observed in the SW+O+N treatments amended with S-EW or S-NS, and as reported in previous studies (Kleindienst *et al.*, 2015a; Chapters II and III of this thesis). However, it should be noted that this does not always occur, since some studies (e.g. Passow *et al.* 2017) showed that the addition of dispersant Corexit 9500A to oil-contaminated seawater leads to a reduction in the formation of MOS – though this study focused on MOS formation by diatoms. The authors of that study showed that the aggregation rate of diatoms is higher in the presence of undispersed oil, and which decreased drastically when Corexit was applied; hence, diatom aggregation, and thus MOS formation, seemed to be inhibited by the presence of the Corexit. These somewhat contradicting results across studies reflect the need to conduct these types of experiments in different marine water bodies where oil spills might be predicted to occur.

#### **4.6.3. Bacterial community response to oil and/or dispersant in seawater during the winter of December 2015**

Oil degrading bacterial communities are commonly present in marine environments and, depending on various factors, will degrade different hydrocarbon constituents that comprise different oils (Orcutt *et al.*, 2010; Kleindienst *et al.*, 2015a; Chapter II of this thesis). In the present study, an enrichment of these types of bacteria was also found in the study conducted that comprised Chapter II of this thesis. In the treatments of SW+O+N amended with S-EW or S-NS, families that contain members with reported hydrocarbon-degrading capabilities were identified – these included

*Halomonadaceae*, *Alteromonadaceae* *Rhodobacteraceae* and *Vibrionaceae* – and which are also commonly associated with producing EPS (Arias *et al.*, 2003; Mancuso Nichols *et al.*, 2004; Bhaskar and Bhosle, 2005; Gutierrez *et al.*, 2007, 2008, 2009, 2013). EPS production is an interesting quality with respect to this study because it has previously been implicated in the formation of MOS (Gutierrez *et al.*, 2013) and MDS (Chapter III of this thesis). Members of the family *Halomonadaceae*, in particular of the genus *Halomonas*, are well known for their ability to produce large quantities of EPS (Quesada *et al.*, 1994; Béjar *et al.*, 1998; Calvo *et al.*, 2002; Arias *et al.*, 2003; Gutierrez *et al.*, 2007), and like for many other EPS-producing marine bacteria that comprise members of the families *Alteromonadaceae* and *Pseudoalteromonadaceae*, they can play an important contribution to the total dissolved organic matter (DOM) pool in the ocean (Azam, 1998).

In the case of the seawater exposed to nutrients and dispersant only, SW+N amended with S-EW or S-NS, the bacterial community composition seemed to have altered compared to that in the SW and SW+N treatments. In the treatment SW+N amended with S-EW, the taxa *Vibrionaceae*, *Oceanospirillales* are present and at the end of the experiment (T<sub>6</sub>), a small proportion (10-15%) of *Rhodospirillaceae* appears. In the case of SW+N amended with S-NS, the same taxa appear in addition to members of the *Gammaproteobacteria*. Interestingly, both profiles strongly differ from the SW+O+N treatment that only contains oil and not dispersant. Although further work is needed, it suggests the possibility that some of these enriched taxa might be involved in degrading these types of dispersants, or even utilising it as a carbon and energy source.

Since the bacterial response observed in the SW+O+N amended with S-NS treatment was more similar to that in the SW+O+N treatment, this could suggest that the application of Slickgone EW modifies the bacterial community more than Slickgone NS and compared to the oil-degrading community response observed with the SW+O+N treatment without addition of any dispersant. However, it would have been very useful to have the hydrocarbon composition of the treatments for this season too, in order to be able to see the effect of those community changes in parallel to biodegradation of the oil. So far, it is only possible to say that the bacterial community seems to vary depending on the dispersant used in the winter (December 2015) and, furthermore, that the type of dispersant has an effect in shaping the bacterial community composition.

In conclusion, it is possible to say that the dispersant S-EW has a stronger effect on the bacterial community composition than S-NS. With reference to the chemical composition of both these dispersants (Tables 4.4 and 4.5), they do differ somewhat. Slickgone NS is reported to contain anionic surfactant and kerosene, while Slickgone EW is composed of kerosene, sodium dioctylsulphosuccinate and ethers of dipropylene glycol. It is important to note that these ingredients within these dispersant formulations are permitted to be public knowledge, and the full details of their composition remains proprietary knowledge to the companies that produce them.

#### **4.6.4. Bacterial community response to oil and/or dispersant in seawater during the autumn of October 2017**

Due to the high primary productivity (based on the high chlorophyll-a concentrations) in the FSC when the autumn experiment (October 2017) was performed, it can be assumed that this provides autochthonous bacterial communities with an available source of macro and micro nutrients. A strong, positive relationship between bacterial abundance and chlorophyll a has previously been reported in marine systems (Bird and Kalff, 1984; White *et al.*, 1990). This is confirmed by the fact that in this study, the bacterial community composition does not significantly change during this season across treatments, nor within the timeframe of these experiments, and remains quite similar to the SW treatment from when these experiments were commenced (at T<sub>0</sub>). This suggest that the bacterial community is defined by the high amount of potential sources of carbon and stays similar and diverse, and is not influenced by enrichment with crude oil and/or dispersant. Moreover, it is well known that bacterial growth rates and metabolism increase with increasing temperatures (e.g. Price and Sowers, 2004). In October 2017 the temperature of surface waters in the FSC was higher, albeit by a couple degrees, compared to in the winter. Hence, a higher metabolic activity of the bacterial communities could be expected than in the winter when their metabolic rates would be relatively lower. This may be translated to explain why no significant change or succession in the bacterial community profile was observed over the durations of these experiments through time. This could be explained by the fact that the C source used is rapidly used by the bacteria, thereby becoming undetectable. Also, the possible high availability of carbon leads to the idea that there is no need to specialise nor compete for any of the added oil and/or dispersant.

The treatments of SW+O+N amended with S-EW or S-NS contained members with reported hydrocarbon-degrading capabilities, such as *Alcanivoraceae*, *Pseudoalteromonadaceae*, *Rhodobacteraceae* and *Rhodospirallaceae*, some of which are also commonly associated with producing EPS (Arias *et al.*, 2003; Mancuso Nichols *et al.*, 2004; Bhaskar and Bhosle, 2005; Gutierrez *et al.*, 2007, 2008, 2009, 2013). This suggest that during this season, the bacterial community composition related to the presence of only dispersant is similar to the one in presence of only oil. In this season, there is no difference in bacterial community composition with the use of one or the other dispersant. This could be linked to the fact that in October 2017, the chlorophyll a and nutrient concentrations were sufficient to support these communities without the need to turn their metabolic interests towards using oil and/or dispersant as a carbon and energy source. Alternatively, temperature could have played a role in this respect. Furthermore, if these experiments had been allowed to run for longer, a marked change or succession in the bacterial community profiles may have been observed, such as an enrichment in taxa that are specialised in the degradation of hydrocarbons.

In the case of the SW+O+N amended with S-EW or S-NS, the bacterial community composition were similar, with a predominance for members of the families *Alteromonadaceae*, *Colwelliaceae*, *Flavobacteriaceae*, *Oceanospirillales*, *Pseudoalteromonadaceae* and *Rhodobacteraceae* (15%-20%). This suggests that comparing these treatments with dispersant with the treatments in the presence of only oil, the two different dispersants seem to have the same effect on the bacterial community composition during this season. However, this contradicts the findings of the winter season (see section 4.6.5 of this chapter).

#### **4.6.5. Comparison of the bacterial community composition between seasons**

The bacterial community composition profiles across both seasons (December 2015 and October 2017) were quite different. As previously mentioned (section 4.6.1), only the SW treatment at the commencement of the experiment ( $T_0$ ) was already initially different for each of the two seasons; albeit only one replicate was available for this time point. This difference could probably be due to differences in temperature, macro- and micro-nutrient availability between the two seasons.

Whilst the bacterial community profiles over time for the December 2015 seawater showed marked differences between treatments and changes to the community over time, this was not the case for the October 2017 seawater experiment. This could be

explained by a higher chlorophyll a concentration in autumn than in winter – as a proxy for phytoplankton biomass and thus representing a source of organic carbon and inorganic nutrients during the ‘bust’ of a bloom – and by the fact that in autumn the nutrient availability is expected to be higher than in winter in the FSC as previously reported (e.g. Gould *et al.*, 1985; Berx *et al.*, 2013). To elaborate, during the winter months (c.f. December 2015), bacteria may not have much in the way of options to support their growth other than oil when it becomes available in the event of a spill, and possibly also dispersant if used. The converse might be expected during the autumn months (c.f. October 2017) where high concentrations of nutrients are present in surface waters of the FSC, so the entry of crude oil (and dispersant if used) might not have much impact on the bacterial community. However, oil-degrading bacteria are almost always enriched in marine environments during the entry of crude oil (or its petrochemical derivatives), thus suggesting that temperature, and/or another as yet unknown environmental factor(s), may have had a key role in structuring the bacterial communities in these experiments performed to compare these two seasons.

In treatments with seawater from December 2015 and amended solely with either one of the dispersants (S-EW or S-NS), the response of the bacterial community appears to be related to the presence of the dispersant since the community was quite different to the one in the SW, SW+N and SW+O+N treatments. The community in these treatments amended with just dispersant was represented by families with members with recognised oil-degrading capabilities, such as *Colwelliaceae*, *Pseudoalteromonadaceae* and *Oceanospirillaceae*. This confirms previous statements and the chemical composition of the dispersants, that seem to contain hydrocarbons or chemical similar structures (such as kerosene), which leads to the presence of an oil-degrading bacteria community. Also, it seems that the chemical composition of the dispersants (Tables 4.4 and 4.5) is different enough to shape the bacterial community in a different way.

Furthermore, the type of dispersant used (S-EW or S-NS) enriched for different taxa even with the presence of oil. For example, in SW+O+N amended with S-EW, the community became strongly dominated by the family *Vibrionaceae*, which contains members with recognised oil-degrading abilities; members of this family were not identified in this same treatment for the October 2017 experiment. Other taxa were also identified in the winter 2015 experiment that were not identified in the autumn 2017 experiment. Kleindienst *et al.* (2015b) suggested that some bacteria would rather prefer

to degrade the dispersant first than the oil. Whether this was the case with the taxa observed to have been enriched in the dispersant-amended treatments warrants further investigation. This could be due to a simpler chemical structure of the dispersant easier to break up by the bacterial activity than the hydrocarbons. Moreover, it is demonstrated (e.g. Chapter III of this thesis) that the amendment by dispersant enhances EPS production from bacteria, and this could lead to an enhancement and stimulation of biodegradation and bacterial activity (Iwabuchi *et al.*, 2002; Calvo *et al.*, 2008). It is well known that EPS can act as a biosurfactant, increasing the solubility of hydrophobic compounds and bioavailability (Vasconcellos *et al.*, 2011). This suggests that the use of dispersant makes the source of carbon present in the oil more available to the bacterial community.

#### **4.6.6. Hydrocarbon analysis October 2017**

Among the environmental factors known to limit biodegradation of petroleum hydrocarbons in the marine environment, nutrient availability is among the most important (Atlas and Bartha, 1972; Head and Swannell, 1999). Depending on the composition of the crude oil and quantities released, its effects on living organisms and its fate in the environment can be quite variable (Atlas, 1975). As shown in Figure 4.9, it is intriguing, and unexpected, that the peak areas of the alkanes C<sub>18</sub> and C<sub>20</sub> are higher at T<sub>6</sub> than at T<sub>4</sub>. This is difficult to explain at present, but one reason could be that the presence of dispersant, and more specifically hydrocarbon constituents of the dispersant formulae, may have contributed directly, or indirectly via formation of hydrocarbon degradation products, with retention times that coincided exactly to the C<sub>18</sub> and C<sub>20</sub> alkanes at those specific time points.

Generally, aliphatic compounds are more amenable to biodegradation than aromatic compounds. It is common to observe the degradation of the former quite early at the onset of an oil spill at sea with a sequential degradation of the aromatic fraction (Head *et al.*, 2006) – in the case of PAHs, the higher the number of rings confers them with a greater recalcitrance to biodegradation (Nzila *et al.*, 2018). In the case of the treatments SW+O+N and SW+O+N amended with S-EW, the aliphatic compounds (*n*-alkanes) started to be biodegraded by the bacterial community, whereas this did not occur in the SW+O+N treatment amended with S-NS. This is an interesting result as it shows that the dispersant S-EW affected the biodegradation of the oil, which was not observed with the other dispersant (S-NS). With the former, the short-chain alkanes are more abundant than the long chain alkanes, and certain members of the bacterial community are being able to degrade long chain *n*-alkanes into shorter ones. This results in an



increase of C<sub>17</sub>, C<sub>18</sub>, C<sub>19</sub> and a decrease in chains of >C<sub>20</sub>. However, the fact that with Slickgone NS, a pattern of n-alkanes biodegradation is not observed, this could mean that there is an impact Slickgone NS on the biodegradation of the oil inhibiting or slowing down the biodegradation process. In the presence of Slickgone NS, the shorter chain n-alkanes present a very low peak area, suggesting that the microorganisms were less or not able to metabolise branched alkanes during the first six weeks of the experiment. This suggests that different dispersants have a differential effect on oil biodegradation, and the same applies in affecting MOS formation as discussed above.

This is significant as it shows that not all dispersants that are approved by the UK, and that are stockpiled worldwide for use in oil spills, will work effectively for combatting spills anywhere at sea. All dispersants should effectively be evaluated for their performance, not only with all different types of crude oils (as they already are), but to also do this at geographically different ocean environments. It would also be useful to know the exact chemical composition of the dispersants, as such information for this is quite vague (Tables 4.3 and 4.4) due to proprietary reasons; it would be useful to understand which chemical constituent(s) in dispersant formulations might affect changes in a bacterial response to oil during a spill, and that might also impact biodegradation rates and the extent of biological degradation. A collaboration between the dispersants producers and the scientific researchers would allow a better response and contingency plans in response to a potential oil spill when having to choose a dispersant.

In this study, measurements of alkane and aromatic hydrocarbon analysis were done, which showed either suppression or stimulation of oil biodegradation in the presence of dispersants. This was dependent on the dispersant used, as in this case S-EW biodegradation of the oil was enhanced. With respect to no detection of biodegradation for the aromatic fraction, this may be explained because these experiments were run for no longer than 6 weeks; a longer run time may have resulted in biodegradation of these hydrocarbon species.

In order to have a more complete dataset, it would have been beneficial to have performed the hydrocarbon analysis to assess biodegradation for the December 2015 experiment. This would have allowed a comparison of the biodegradation profiles of the bacterial communities for both seasons in parallel with the microbial community response profiles.

#### **4.7. Conclusions**

Whilst results from laboratory experiments cannot be directly linked to *in situ* conditions, they provide insights that improve the capacity of prediction and enhanced environmental assessment. This study showed that MOS and MDS aggregates can form after the amendment by oil and/or dispersant at two different seasons (autumn and winter) independently of the seawater properties belonging to the same water mass. Secondly, seasonality (nutrients, chlorophyll a and temperature) seem to shape the bacterial community composition that seems different between both seasons. Thirdly, the type and chemistry of the dispersant, here between Slickgone NS and Slickgone EW, applied to an oil spill case appears to have a detrimental effect on the bacterial community composition leading to a potential difference in oil-biodegradation effect. With respect to the experiment conducted using seawater from October 2017, it is possible to say that with the amendment of the S-EW (Slickgone EW) a normal biodegradation pattern of *n*-alkanes occurred. However, in the case of applying S-NS (Slickgone NS) no indication of biodegradation occurred. These considerations should be considered and further explored to inform better decision making for selecting a dispersant type that will have the maximum effect for enhancing oil biodegradation, and where possible balanced against reducing the environmental impact.



## **Chapter V- Synthesis and future work**

## **5.1. Overview**

The overall objective of my PhD was to study MOS formation and the potential factors participating in its formation in sea surface waters of the FSC in the event of an oil spill in this region. This purpose of this final chapter is to summarise the main findings of the research that comprises this thesis, as well as to discuss additional work that is presented in Appendix D that is related to this thesis research, but which was decided to not include into the three main results chapters (Chapters II, III and IV). Finally, recommendations for future work are also discussed.

## **5.2. Chapter summaries**

### **5.2.1. Chapter I: Introduction- Sources, Fate and Response to Oil Spills in the Faroe-Shetland Channel: A Microbiological Perspective**

The aim of Chapter I was to introduce the FSC, which was the focus marine region for the research conducted in this thesis, and to provide a general overview on the potential fate of crude oil in the event of oil contamination in the marine environment, including the formation of MOS and the response of oil-degrading bacterial communities in the presence versus absence of when chemical dispersants are used to combat oil spills at sea. Some notable highlights from this literature review are:

- (1) The FSC is a deepwater offshore region that has witnessed in the past few decades a strong development in the oil and gas industry. As it is a highly hydrodynamic area, with relatively strong currents that run in opposite directions depending on depth, future deepwater oil extraction in this region is likely to pose enormous challenges with respect to combatting a major spill in this region. An abiotic control could have been run to see if existing EPS would have precipitated.
- (2) Through weathering processes, oil can be transformed in several different ways after its entry into the marine environment, such as wind, photo-oxidation, bio-degradation, precipitation to the seafloor through MOS formation. The characteristics of the region would determine the fate and ultimate end point of the oil, as indeed also its impact(s) to the surrounding ecosystem.
- (3) MOS has been shown to form after some oil spills, but not all, and its ultimate fate is recognised to be the seafloor via its transport precipitation after its formation on the sea surface and upper water subsurface layers.

- (4) Oil-degrading bacteria play a fundamental role in the biodegradation of hydrocarbons that constitute crude oil, thus providing a key tool for bioremediation strategies. Such organisms have been identified in marine environments following oil or petrochemical contamination, including during the more recent DWH oil spill (Gutierrez *et al.*, 2013; Kleindienst *et al.*, 2015a; Yang *et al.*, 2016).
- (5) Chemical dispersants are commonly one of the first tools that are used to combat oils spills at sea, although significant conjecture surrounds their use due to their potential to be toxic to marine organisms, including to inhibit certain members of the oil-degrading bacterial community in seawater. Effects of chemical dispersants on MOS formation, and to marine fauna, are still quite poorly understood.

#### **5.2.2. Chapter II: Role of EPS, Dispersant and Nutrients on the Microbial Response and MOS Formation in the Subarctic Northeast Atlantic**

MOS formation was observed previously after some oil spill disasters such as the DWH (Passow, 2014; Kleindienst *et al.*, 2015a). The aim of Chapter II was to present the first study examining MOS formation in surface waters of the FSC. This is important considering that the FSC has witnessed over the last decades an increase in oil exploitation and transportation activities. Some of the main outcomes of this Chapter are summarised as follows:

- (1) In the event of an oil spill in the FSC, the use of dispersants would likely lead to the formation of MOS and lead to a subsurface “dirty blizzard,” reminiscent to that during the DWH oil spill where a large proportion of sea surface oil ended up on the seafloor. A MOS “fall out” could lead to detrimental impacts to benthic ecosystems in the FSC, such as sponge belts that are common in this channel of the Atlantic.
- (2) In the absence of dispersant applications, MOS did not form so it is expected that the majority of surface oil is likely to remain at the sea surface where it could potentially impact coastal regions, such as the Faroe and Shetland isles, and northern coastal regions of Scotland.
- (3) MOS particles harbour rich communities of prokaryotes, including oil-degrading bacteria. Thus, MOS that forms in the FSC could potentially act as ‘hot spots’ where a heightened level of oil biodegradation occurs, although further work would be needed to assess this.

- (4) The bacterial community response in experiments using seawater from the FSC amended with crude oil and with/without chemical dispersant mirror that observed during the DWH, and hence underscore their broad relevance.

### **5.2.3. Chapter III: Chemical dispersant enhances microbial exopolymer (EPS) production and formation of marine oil/dispersant snow in surface waters of the subarctic northeast Atlantic**

The aim of Chapter III was to investigate the importance of EPS in MOS and MDS formation. A major highlight from this work was that the application of chemical dispersants can enhance EPS production by bacteria. This and some other major findings from this chapter are summarised as follows:

- (1) During exposure of FSC sea water to a chemical dispersant, whether in the presence/absence of crude oil, the dispersant stimulates the production of significant quantities of EPS that is a key structural component of MOS. It is suspected that EPS plays a major role in MOS formation, and likely conferred via de-novo synthesis of EPS by natural communities of EPS-producing bacteria.
- (2) The formation of MDS seems to be a product of adding chemical dispersants to seawater and forms independently of the presence/absence of oil. Its impact to the marine ecosystem remains poorly understood since MDS was first reported in the literature only recently from the work in chapter II (see also Suja *et al.*, 2017).
- (3) This chapter reported, for the first time, the bacterial communities associated with MDS aggregates using Illumina MiSeq sequencing technology to reveal that their diversity is not significantly dissimilar to those associated with MOS aggregates.

### **5.2.4. Chapter IV: Effect of two different chemical dispersants on the biodegradation and bacterial response to crude oil**

The aim of Chapter IV was to assess two different chemical dispersants on the bacterial community response to crude oil at two different seasons (autumn and winter) in experiments using surface water from the FSC. Some of the main findings from this work are summarised as follows:

- (1) Seasonality does not seem to affect MOS and MDS formation in sea surface waters of the FSC. Both formed irrespective of the season in this case, as indeed irrespective of the chemical dispersant used.

- (2) In October 2017, the chemical dispersant Slickgone EW (S-EW) effected a typical community response, with oil-degrading bacteria observed to have become enriched, and which was indicative of biodegradation of the oil as measured by oil analysis. In contrast, use of the chemical dispersant Slickgone NS (S-NS) did not result in any significant biodegradation of the oil. These results clearly indicate that different dispersants can result in a completely different response by the bacterial community.
- (3) Surface waters of the FSC showed differences in some physicochemical properties during the autumn compared to winter months, which might help explain some of the differences observed in the bacterial community response across the two seasons assessed.
- (4) These considerations should be taken into account and further explored to inform decision making for selecting a dispersant type that will have maximum effect for enhancing oil biodegradation, and where possible balanced against reducing environmental impact.

### **5.3. Additional work conducted during the PhD**

Some extra work presented in the Appendices A-D of this thesis was done during my PhD. Appendix B shows an explanation of the preliminary experiments for Chapter II. Those experiments were not successful so they were not included in any of the chapters.

In the Appendix A, other studies and collaborations conducted during the period of my PhD are cited, as well as the impact of this research in various media portals (newspapers, radio news etc.). In Appendix D, other preliminary work is presented that is also related to my research. This work describes potential factors influencing the formation of MOS in surface and deep waters of the FSC. Firstly, more work will be needed to assess whether quorum sensing mechanisms could play a role in MOS formation and in structuring the bacterial community associated with these aggregates. Quorum sensing is a communication signal molecule between bacteria that acts through the ability to detect and to respond to cell population density by gene regulation. The higher the concentration of the quorum signal molecule, the greater the cell population, and which could enhance MOS formation. The data presented in Appendix D is preliminary, but might suggest that quorum sensing has some bearing in MOS formation, but which has yet to be further investigated.



#### **5.4. Conclusion**

The fundamental ecological importance of the microbial communities as the base of the food chain and their recognised ability for oil-degradation in the oceans has spurred a lot of interest as a potential way to harness their potential for the bio-remediation of environments contaminated with oil (Yakimov *et al.*, 2007; Atlas and Hazen, 2011). As documented in other cases (e.g. DWH, and *Tsesis* oil spills), in the event of a spill at sea the formation of MOS is an important process leading to the transportation of the oil from the upper water column to the seafloor. The work conducted during my PhD project put together different studies leading to a better knowledge and understanding of how the ecosystem responds in case of an oil spill in the FSC waters: MOS formation in the FSC and factors enhancing its production.

Chapter II describes for the first time the MOS formation in the surface seawaters of the FSC with the presence of nutrients and dispersants. In the event of an oil spill in the FSC, the use of dispersants would likely lead to the formation of MOS and trigger similar consequences to that during the DWH oil spill where a large proportion of sea surface oil ended up on the seafloor (e.g. Almeda *et al.*, 2014). This thesis forms the first body of work revealing that MOS can form in the FSC in the event of an oil spill. Moreover, this chapter highlights the role of nutrients and dispersant in MOS formation, as also reported in other studies (e.g. Kleindienst *et al.*, 2015). This chapter also showed that the MOS particles that formed harboured diverse communities of prokaryotes, including oil-degrading bacteria; it is assumed that these particles potentially act as ‘hot spots’ where a heightened level of oil biodegradation could occur in the marine water column during a spill. Furthermore, MOS is shown here to form in the FSC, which is a contrasting water body to that of the Gulf of Mexico where MOS had also been observed to form during oil spillage. I surmise that MOS could possibly form anywhere in the event of an oil spill, however could depend on the physicochemical conditions (e.g. seasonality, currents) of the seawater, and especially likely the presence of nutrients and/or use of chemical dispersants.

In Chapter III, different conclusions were made. Firstly, that EPS concentrations in seawater are strongly increased as a response to when a chemical dispersant is applied, and interestingly occurred irrespective of whether crude oil is present or not. This is likely due to EPS-producing bacteria, and while it has only been described in Chapters II and III, dispersant seems to be crucial in the formation of MOS. Secondly, this chapter also shows that in the event of an oil spill in the FSC, the use of chemical dispersants would

likely lead to the formation of MOS and MDS harbouring a similar bacterial community dominated by hydrocarbon degrading and EPS producing bacteria. MDS formation (in the absence of crude oil) has been observed in laboratory-based experiments (e.g. Suja *et al.*, 2017), however, understanding how it forms, what factors promote this and its impacts to marine organisms is deemed important for oil-spill response authorities to decide whether a chemical dispersant should be used in the event of a spill at sea. Thus, future investigations in this respect should consider MDS formation, including its fate and impacts.

Finally, Chapter IV evaluated the effect of two different dispersants on MOS formation and the bacterial community response over two different seasons of the year. Firstly, MOS and MDS were formed at both seasons independently of the dispersant used. Secondly, seasonality seemed to shape the bacterial community composition. Thirdly, it was possible to observe a different bacterial community composition in December 2015 with the use of one or the other dispersant. Although having a similar chemical composition, the small differences observed could have repercussions to the overall oil biodegradation process. In October 2017, the bacterial community composition was not found to vary with the use of the two different dispersants. However, during this season, the dispersant Slickgone EW resulted in the detectable biodegradation of the oil, while this was not the case with the dispersant Slickgone NS. These results highlight the importance of selecting a chemical dispersant for use in a specific marine environment, as one chemical dispersant cannot be expected to be effective for impacting the enrichment of oil-degrading bacterial communities for effective biodegradation of the oil.

### **5.5. Future work**

The findings of this project offer quite numerous opportunities for further studies. Here, the most relevant ones are described to be hopefully pursued in future studies.

Firstly, the fact that MOS formation in the FSC was described for the first time in Chapter II, this assumes the possibility of creating a model that would predict where MOS could potentially end up in case of an oil spill. Results from this thesis, from further work and integrating oceanographic data could provide enough information to help predict this and what organisms or ecosystems could become impacted in the FSC and its adjacent waterways (e.g. benthos fauna, currents, distance covered, size of area affected etc.). Modelling of the fate of oil after an oil spill has already been done previously, such as for the DWH (Liu *et al.*, 2011; French-McCay *et al.*, 2016) and Bohai China (Xu *et al.*, 2015;

Yu *et al.*, 2016). Some studies have also modelled the fate of the oil in the FSC (Main *et al.*, 2017; Gallego *et al.*, 2018). However, the fate of MOS formed if an oil spill were to occur has not been studied to any great extent yet. There are few studies introducing this idea, such as by Dissanayake *et al.* (2018) who made the first steps into MOS fate modelling after the DWH in the Gulf of Mexico. It would be interesting to model MOS fate in vulnerable areas to anticipate and predict where MOS could end up if an oil spill were to occur. This could lead to a better assessment of the situation, and feed this to improve oil spill contingency efforts.

Secondly, based on the work in Chapters II and III, the study of MDS and its potential consequences on the ecosystem would be an interesting field to develop since it is a factor not taken in account previously due to lack of information. Now, it is known that those dispersant aggregates can form and can also precipitate and modify the bacterial community composition of the ecosystem affected. It would be worthy to extend this work to understand more MDS formation and its impacts.

Thirdly, other factors may influence MOS formation apart from nutrients and dispersants (Chapters II, III, and IV). As seen in some preliminary studies (see Appendix section), light, depth and oxygen availability are factors that require further investigation for their potential effect on MOS formation. This has already been studied in other areas such as the Gulf of Mexico (e.g. Daly *et al.*, 2016; Passow and Ziergovel, 2016). It would be interesting to follow up on this with respect to the FSC. Quorum sensing is another potentially important mechanism that could contribute to MOS formation. It would be very interesting to keep developing this idea as a potential way to further improve bio-remediation strategies to treat oil pollution in marine environments.

Finally, based on the work in Chapter IV, the nature of the dispersant appears to define the bacterial community composition in FSC surface waters. It would be interesting to see how other UK approved chemical dispersants, or even whether bio-surfactants, would affect the bacterial community response to oil, as well as the oil-biodegradation process as this could help the oil and gas industry to select less harmful, and more effective, dispersants. To combine these studies with ecotoxicological studies would be a step in the right direction toward helping to prevent the impacts of oil spillage and use of harmful dispersants with respect to the FSC and other adjacent marine environments.

Generally, a good idea would be to do in situ experiments to see how the ecosystem would really react in case of an oil spill. Some research groups (e.g. Oban) have done some experiments exposing a small volume of water to an oil slick to model the fate of the oil.

However, licences and the approval of the Scottish government are needed due to the possible danger and damage to the environment.

# Appendix A

## **Appendix A- Academic Impact**

### **Collaborations during my PhD**

Hauquier, F., Suja, L. D., Gutt, J., Veit-Köhler, G., & Vanreusel, A. (2015). Different oceanographic regimes in the vicinity of the Antarctic Peninsula reflected in benthic nematode communities. *PloS one*, 10(9), e0137527.

Henry, L. A., Orejas, C., Kazanidis, G., Suja, L. D., Witte, U., & Roberts, J. M. (2016). Coral cities of the deep. *Ocean Challenge*, 21(2).

Duran Suja, L., Vad, J., Henry, L.-A. & Roberts, J.M. (IN PREP). The tunicate *Polycarpa pomaria* (Savigny, 1816), a possible contributor to cold water coral reef stability and ecosystem function. *Eliyon*.

Vad, J., Duran Suja, L., Summers, S., Roberts, J.M. & Henry, T.B. (IN PREP). Effects of crude oil, dispersed crude oil and dispersant contaminated sediments on model sponge *Halichondria panicea*, *Marine Ecology Progress Series*.

Veit-Köhler, G., Durst, S., Schuckebrock, J., Hauquier, F., Suja, L. D., Dorschel, B., ... & Arbizu, P. M. (2018). Oceanographic and topographic conditions structure benthic meiofauna communities in the Weddell Sea, Bransfield Strait and Drake Passage (Antarctic). *Progress in Oceanography*, 162, 240-256.

### **Posters and presentations during my PhD**

-Durán Suja, L., L.-A. Henry & J.M. Roberts (2015). The tunicate *Polycarpa pomaria*, a possible contributor to cold-water coral reef architecture. Poster, Deep-Sea Biology Symposium, Aveiro, Portugal, 30.9–5.9 .2015.

-Durán Suja, L. & Gutierrez T. (2015). “Evaluating the resilience of sea surface and deepwater systems to recover from oil spills in the Faroe-Shetland Channel”. SLS PhD conference, Heriot Watt University, Edinburgh, UK. PRIZE BEST PRESENTATION.

- Durán Suja, L., Summers, S. & Gutierrez, T. (2016). Dispersant-induced marine oil snow formation in the Faroe Shetland Channel and the associated microbial response. CDT in Oil and Gas Annual Conference (7<sup>th</sup> Nov 2016), Heriot Watt University, Edinburgh, UK.

- Durán Suja, L., Summers, S. & Gutierrez, T. (2017). Effect of two chemical dispersants on the microbial community response and MOS formation to crude oil spillage in the Faroe Shetland Channel. Poster, MASTS conference, 4-6.10.2017, Glasgow, UK.

- Durán Suja, L. & Gutierrez T. (2017). "Evaluating the resilience of sea surface and deepwater systems to recover from oil spills in the Faroe-Shetland Channel". CDT in Oil and Gas Annual Conference (3<sup>rd</sup> Nov 2017), Heriot Watt University, Edinburgh, UK.

- Durán Suja, L., Summers, S. & Gutierrez, T.(2018). Effect of two chemical dispersants on the microbial community response to crude oil spillage in the subarctic northeast Atlantic. Poster, ISTME symposium (13-17.8.2018) Leipzig, Germany.

### Media impact of the publications involved in my PhD

**Researchers warn of oil spill complexities**  
*Western Morning News (Devon) (Main), 13/04/2017, p.22, Lucinda Cameron*  
 the Gulf of Mexico in 2010, scientists said. When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in Edinburgh found use of a dispersant in the FSC after an oil spill would trigger conditions similar to the aftermath of Deepwater Horizon and the formation of

**'Dirty blizzard' fears if oil spill hits north-west of UK**  
*The Scotsman (Main), 13/04/2017, p.14, Lucinda Cameron*  
 the Gulf of Mexico in 2010, scientists said. When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in Edinburgh found use of a dispersant in the FSC after an oil spill would trigger conditions similar to the aftermath of Deepwater Horizon and the formation of

**Scientists warn of oil spill risk in Atlantic**  
*The Herald (Main), 13/04/2017, p.8, Unattributed*  
 the Gulf of Mexico in 2010, scientists said. When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in Edinburgh found use of a dispersant in the FSC after an oil spill would trigger conditions similar to the aftermath of Deepwater Horizon and the formation of

**Atlantic oil spill may be very difficult to combat, warns study**  
*The Courier (Main Edition) (Main), 13/04/2017, p.5, Unattributed*  
 the Gulf of Mexico in 2010, scientists said. When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in Edinburgh found use of a dispersant in the FSC after an oil spill would trigger conditions similar to the aftermath of Deepwater Horizon and the formation of

**Atlantic oil spill could repeat Deepwater Horizon 'dirty blizzard', study finds**  
*Herald Scotland (Web) (News), 12/04/2017, p.1, Unattributed*  
the Gulf of Mexico in 2010, scientists said. When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in Edinburgh found use of a dispersant in the FSC after an oil spill would trigger conditions similar to the aftermath of Deepwater Horizon and the formation of

**Atlantic oil spill could repeat Deepwater Horizon 'dirty blizzard', study warns**  
*Stornoway Gazette (Web), 12/04/2017, Unattributed*  
Gulf of Mexico in 2010, scientists said. Marine oil snow comprises sticky, floating organic particles that are visible to the naked eye and contain oil droplets from spills (Heriot-Watt University/PA Wire) When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in

**Atlantic oil spill could repeat Deepwater Horizon 'dirty blizzard', study warns**  
*Donside Piper & Herald (Web), 12/04/2017, Unattributed*  
Gulf of Mexico in 2010, scientists said. Marine oil snow comprises sticky, floating organic particles that are visible to the naked eye and contain oil droplets from spills (Heriot-Watt University/PA Wire) When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in

**Atlantic oil spill could repeat Deepwater Horizon 'dirty blizzard', study warns**  
*Kirriemuir Herald (Web), 12/04/2017, Unattributed*  
Gulf of Mexico in 2010, scientists said. Marine oil snow comprises sticky, floating organic particles that are visible to the naked eye and contain oil droplets from spills (Heriot-Watt University/PA Wire) When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University in

**Atlantic oil spill could repeat Deepwater Horizon 'dirty blizzard', study warns**  
*Selkirk Today (Web), 12/04/2017, Unattributed*  
Gulf of Mexico in 2010, scientists said. Marine oil snow comprises sticky, floating organic particles that are visible to the naked eye and contain oil droplets from spills (Heriot-Watt University/PA Wire) When tackling oil spills, operators often use dispersants which break up the oil and encourage it to degrade naturally. But researchers at Heriot-Watt University

**Atlantic oil spill could repeat Deepwater Horizon 'dirty blizzard', study warns**  
*Falkirk Herald (Web), 12/04/2017, Unattributed*  
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**A disaster worse than BP's Deepwater Horizon could happen in Scotland if we aren't prepared**  
*The National (Scotland) (Web), 12/04/2017, Unattributed*

barrels of oil into the Gulf of Mexico in what then-President Barack Obama called "the worst environmental disaster America has ever faced". Dr Tony Gutierrez, associate professor of microbiology at Heriot-Watt University in Edinburgh, was in the US at the time and has researched the impact of the incident, which polluted the coastline and saw BP fined \$20 billion dollars.

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## **Appendix B**

## **B- Preliminary experiments of Chapter II**

In the Gulf of Mexico, Gutierrez *et al.* (2018) put into practice a protocol where they obtained MOS after few days. As a first attempt to see if MOS would form under the same conditions with seawater from the FSC, few experiments were set up. These preliminary experiments were not successful and helped me to decide to do a change of protocol to carry on my research. The three experiments are briefly described here below due to their simplicity and to the fact that no analysis was conducted due to their failure.

### **1. Filtered and non-filtered oil**

Firstly, in May 2015, two MOS experiment following this protocol on the MRV Scotia cruise were set up and no marine oil snow formation was observed. The same experiment was done during the MRV Scotia in September 2015 cruise to see if the formation of oil snow depended on the season.

The oil of this experiment was filtered and then sterilised through a 0.22µm filter. The aim was to see if the MOS formation came from the bacterial communities from the water column or from the oil itself. These incubations were conducted aboard Scotia during the cruise where 3L of sea surface water (FIM06a, 60° 38.120 N, 4° 54.030 W, Figure 2.2, Chapter II) were collected and put directly into experiment. In a roller-bottle chamber (Figure B.1), the experiment accommodated 6 x 250-mL glass screw-cap bottles, used to set up 3 experiments, each in duplicate, using the 6 glass bottles:

- Two bottles with sea water (controls)
- Two of the bottles were enriched each with approx. 1mL of the crude oil.
- Two other bottles were treated with some Na-azide (0.1g) in order to inhibit microbial growth/ respiration and 1mL of the crude oil.

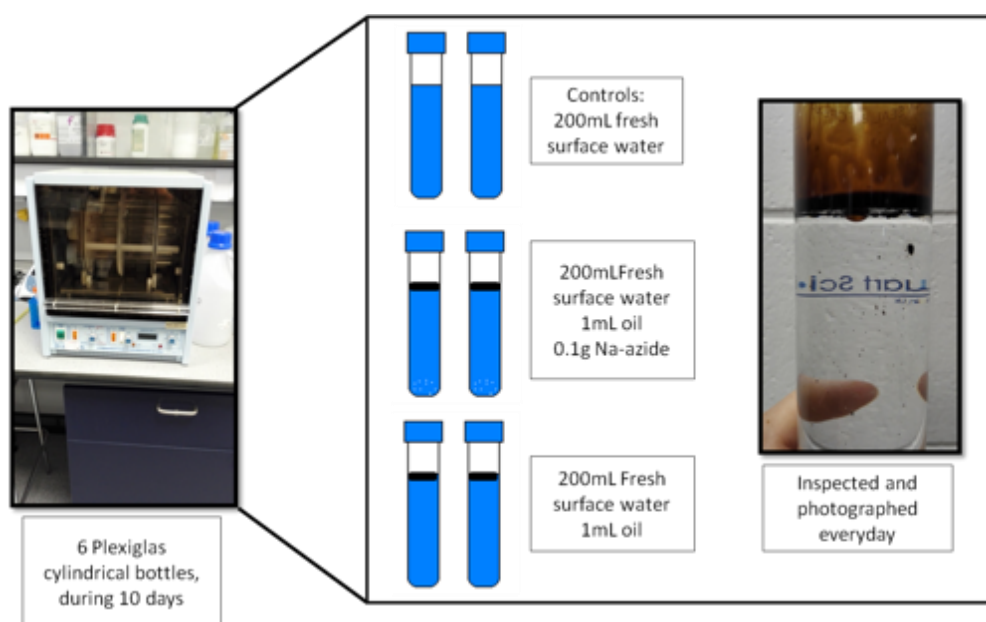
The bottles rotated in the roller-bottle device (Figure B.1) on-board Scotia for ten days and monitored for the formation of MOS.

The experiment resulted in any evidence of Marine Oil Snow formation. It seems that under these conditions, MOS is not likely to form with FSC seawater independently of the season. Moreover, the bacterial community of the oil was removed so maybe this could be another reason why MOS did not form.

Secondly, in this next experiment done in the lab, non-filtered oil was used and thus that means that the oil may have its own bacterial community. This experiment would

have allowed to see if the bacterial community from the oil itself has a role in this MOS formation or if both bacterial communities (from the water column and from the oil) have to be present for MOS formation. These samples were transported intact from the boat to the lab without being opened in any moment. A new experiment is realized in the lab with the same bottles and under the same conditions. Under sterile conditions, bottles were opened and oil was removed (without taking out water from the bottles). New oil was added to the same tubes, this time the oil was not filtered. No MOS was produced nor observed. This experiment could have been improved by doing a whole new experiment from the beginning and not changing one oil by the other.

## 2. Photo-oxidation



**Figure B.1:** Standard MOS experiment design used as preliminary experiment.

Finally, once this experiment done, another one was settled. During the same cruise, 2L of fresh seawater was taken at the same place. That water was being used to set up a new MOS experiment but this time with filtered Schehallion crude oil that was 2 weeks exposed to sunlight (at the window). It is well known that the sunlight may participate in the physical/chemical degradation of the oil (photo-oxidation) and increase the oil surface available for oil degrading bacteria. This factor could be determinant for the formation of Marine Oil Snow due the increase in availability for microorganisms. However, no MOS was produced.



After several trials with these preliminary experiments, I decided to face another protocol including the use of nutrients and dispersants as they have been described in the literature as having a key role in MOS formation. The rest of the chapter describes the successful MOS formation with the use of nutrients and dispersants with seawater from the FSC following Kleindienst *et al.* (2015a).

## Appendix C

**Table 1.** Average Shannon-Wiener Index of each treatment and time points as well as their corresponding standard deviation between replicates. Season December 2015.

<b>Sample</b>	<b>H'</b>	<b>Stdev</b>
SW_T0	2.433	0.001
SW_T2	2.616	0.001
SW_T4	2.097	0.001
SW_T6	2.1705	0.1285
SW+N_T2	2.465	0.044
SW+N_T4	2.494	0.001
SW+N_T6	2.54	0.001
SW+N+S-EW_T2	2.243	0.001
SW+N+S-EW_T4	2.432	0.275
SW+N+S-EW_T6	2.1705	0.0055
SW+N+S-NS_T2	2.2375	0.0245
SW+N+S-NS_T4	2.219	0.009
SW+N+S-NS_T6	2.311	0.004
SW+O+N_T2	2.061	0
SW+O+N_T4	2.545	0.004
SW+O+N_T6	2.2495	0.0145
SW+O+S-EW_T2	2.668	0.004
SW+O+S-EW_T4	2.5285	0.0235
SW+O+S-EW_T6	2.538	0.007
SW+O+S-NS_T2	2.386	0.004
SW+O+S-NS_T4	2.592	0.009
SW+O+S-NS_T6	2.407	0.013
SW+O+N+S-EW_T2	2.187	0
SW+O+N+S-EW_T4	1.993	0.017
SW+O+N+S-EW_T6	2.032	0.004
SW+O+N+S-NS_T2	2.356	0.012
SW+O+N+S-NS_T4	2.364	0.003
SW+O+N+S-NS_T6	2.395	0.011

**Table 2.** Average Shannon-Wiener Index of each treatment and time points as well as their corresponding standard deviation between replicates. Season October 2017.

<b>Treatment</b>	<b>H'</b>	<b>STDEV</b>
SW_T0	3.307	0
SW_T2	3.087	0.253
SW+N_T2	3.537	0.084
SW+N_T4	2.806	0.291
SW+N_T6	2.4885	0.7025
SW+N+S-EW_T2	3.5055	0.1265
SW+N+S-EW_T4	2.688	0
SW+N+S-EW_T6	3.057	0.234
SW+N+S-NS_T2	2.214	2.214
SW+N+S-NS_T4	3.522	0
SW+N+S-NS_T6	2.975	0.542
SW+O+N_T2	2.665	0.085
SW+O+N_T4	2.482	0.265
SW+O+N_T6	2.5995	0.1335
SW+O+S-EW_T2	3.176	0
SW+O+S-EW_T4	2.058	0.481
SW+O+S-EW_T6	2.5895	0.2295
SW+O+S-NS_T2	2.739	0
SW+O+S-NS_T4	2.665	1.013
SW+O+S-NS_T6	3.151	0.201
SW+O+N+S-EW_T2	2.875	0
SW+O+N+S-EW_T4	2.612	0
SW+O+N+S-EW_T6	2.766	0.423
SW+O+N+S-NS_T2	3.065	0.03
SW+O+N+S-NS_T4	3.5165	0.0945
SW+O+N+S-NS_T6	3.322	0.145

**Table 3.** Pr/C17 ratio of the Schiellalion oil and the treatments SW+O+N, SW+O+N+S-EW and SW+O+N+S-NS in the experiment October 2017 and their corresponding standard deviation on the right table. On the left table, Kruskal-Wallis analysis result by factor.

Treatment	Pr/C17	STDEV
Oil	0.039405	0.001913
SW+O+N_T2	0.068705	0.026384
SW+O+N_T4	0.015233	0.003313
SW+O+N_T6	0.026621	0.025583
SW+O+N+S-EW_T2	0.061915	0.005496
SW+O+N+S-EW_T4	0.008615	0.008615
SW+O+N+S-EW_T6	0.052373	0.047627
SW+O+N+S-EW_T2	0.029674	0.028746
SW+O+N+S-EW_T4	0.183935	0.117508
SW+O+N+S-EW_T6	0.076456	0.009234

Factor	Kruskal-Wallis p-value
Time	0.5744
Replicate	0.4057
Treatment	0.0257 *

**Table 4.** Ph/C<sub>18</sub> ratio of the Schiellalion oil and the treatments SW+O+N, SW+O+N+S-EW and SW+O+N+S-NS in the experiment October 2017 and their corresponding standard deviation on the right table. On the left table, Kruskal-Wallis analysis result by factor.

Treatment	Ph/ C <sub>18</sub>	STDEV
Oil	4.970189	0.690189
SW+O+N_T2	6.770803	0.006098
SW+O+N_T4	3.034375	0.965625
SW+O+N_T6	3.562454	1.75293
SW+O+N+S-EW_T2	3.248168	1.367216
SW+O+N+S-EW_T4	3.944703	3.944703
SW+O+N+S-EW_T6	0.720622	0.279378
SW+O+N+S-EW_T2	0.006552	0.003833
SW+O+N+S-EW_T4	0.038307	0.025631
SW+O+N+S-EW_T6	1.061049	0.155167

Factor	Kruskal-Wallis p-value
Time	0.8335
Replicate	0.7624
Treatment	0.4568

**Table 5.** 9MP/1MP ratio of the Schiellalion oil and the treatments SW+O+N, SW+O+N+S-EW and SW+O+N+S-NS in the experiment October 2017 and their corresponding standard deviation on the right table. Non-significant Kruskal-Wallis  $p=0.2402>0.05$ .

<b>Treatment</b>	<b>9MP/1MP</b>	<b>STDEV</b>
Oil	1.710997	0.091564
SW+O+N_T2	1.213314	0.213314
SW+O+N_T4	1	0
SW+O+N_T6	1.49905	0.077932
SW+O+N+S-EW_T2	1.301193	0.301193
SW+O+N+S-EW_T4	1	0.077932
SW+O+N+S-EW_T6	1.58166	0.079679
SW+O+N+S-NS_T2	1	0
SW+O+N+S-NS_T4	1.520033	0
SW+O+N+S-NS_T6	1.548042	0.036813

**Table 6.** 3MP/2MP ratio of the Schiellalion oil and the treatments SW+O+N, SW+O+N+S-EW and SW+O+N+S-NS in the experiment October 2017 and their corresponding standard deviation on the right table. Non-significant Kruskal-Wallis  $p=0.2585>0.05$ .

<b>Treatment</b>	<b>3MP/2MP</b>	<b>STDEV</b>
Oil	0.973252	0.021298
SW+O+N_T2	0.986868	0.013132
SW+O+N_T4	1	0
SW+O+N_T6	0.933132	0.000472
SW+O+N+S-EW_T2	0.97505	0.02495
SW+O+N+S-EW_T4	1	0
SW+O+N+S-EW_T6	0.953177	0.021707
SW+O+N+S-NS_T2	1	0
SW+O+N+S-NS_T4	0.986159	0
SW+O+N+S-NS_T6	0.946746	0.01217

**Table 7.** P/9MP ratio of the Schiellalion oil and the treatments SW+O+N, SW+O+N+S-EW and SW+O+N+S-NS in the experiment October 2017 and their corresponding standard deviation on the right table. Non-significant Kruskal-Wallis  $p=0.4457>0.05$ .

<b>Treatment</b>	<b>9MP/1MP</b>	<b>STDEV</b>
Oil	1.675669733	0.056236
SW+O+N_T2	1.213313518	0.213314
SW+O+N_T4	0.5	0.5
SW+O+N_T6	1.499050256	0.564117
SW+O+N+S-EW_T4	1.661338974	0.03
SW+O+N+S-EW_T6	1.501980911	0.0563
SW+O+N+S-NS_T2	1	0
SW+O+N+S-NS_T4	1.520032638	0.2
SW+O+N+S-NS_T6	1.548042041	0.036813

**Table 8.** 2MN/1MN ratio of the Schiellalion oil and the treatments SW+O+N, SW+O+N+S-EW and SW+O+N+S-NS in the experiment October 2017 and their corresponding standard deviation on the right table. Non-significant Kruskal-Wallis  $p=0.1437>0.05$ .

<b>Treatment</b>	<b>2MN/1MN</b>	<b>STDEV</b>
Oil	3.731268	0.003098
SW+O+N_T2	2.40714	0.632758
SW+O+N_T4	3.715408	1.857704
SW+O+N_T6	4.289344	0.766342
SW+O+N+S-EW_T2	3.363458	0.061852
SW+O+N+S-EW_T4	2.088068	1.363714
SW+O+N+S-EW_T6	3.784114	1.892057
SW+O+N+S-NS_T2	3.119982	0.194234
SW+O+N+S-NS_T4	3.260398	0.0065
SW+O+N+S-NS_T6	3.271587	0.225234

## Appendix D



## **Appendix D- Quorum sensing as a possible factor influencing the formation of MOS**

### **Overview**

In this chapter annexed to this thesis, preliminary laboratory experiments have been performed in constant agitation to simulate natural sea conditions and incubated under different conditions to investigate potential parameters involved in MOS formation (Shanks and Edmondson, 1989; Passow, 2016). Detailed information related to the physical and chemical conditions influencing MOS formation, as well as what processes are involved in this process, is quite limited. It is divided in two sections, one studying the quorum sensing as a potential factor involved in MOS formation, and then a section addressing few other potential factors.

### **1. Introduction**

As mentioned previously, studies have shown that MOS is a product of an interaction between suspended organic matter and oil (Fu *et al.*, 2014), and that its formation could potentially enhances oil degradation by the MOS-associated community of marine oil degrading bacteria, likely by increasing the bioavailability of the oil to the bacteria (Atlas and Hazen, 2011). The underlying mechanism(s) that affect the formation of MOS are not fully understood. Factors such as hydrodynamic conditions, collision rate of suspended particles, particle coagulation and flocculation and interaction of oil components with microorganism may be important in this process (Passow *et al.*, 2012).

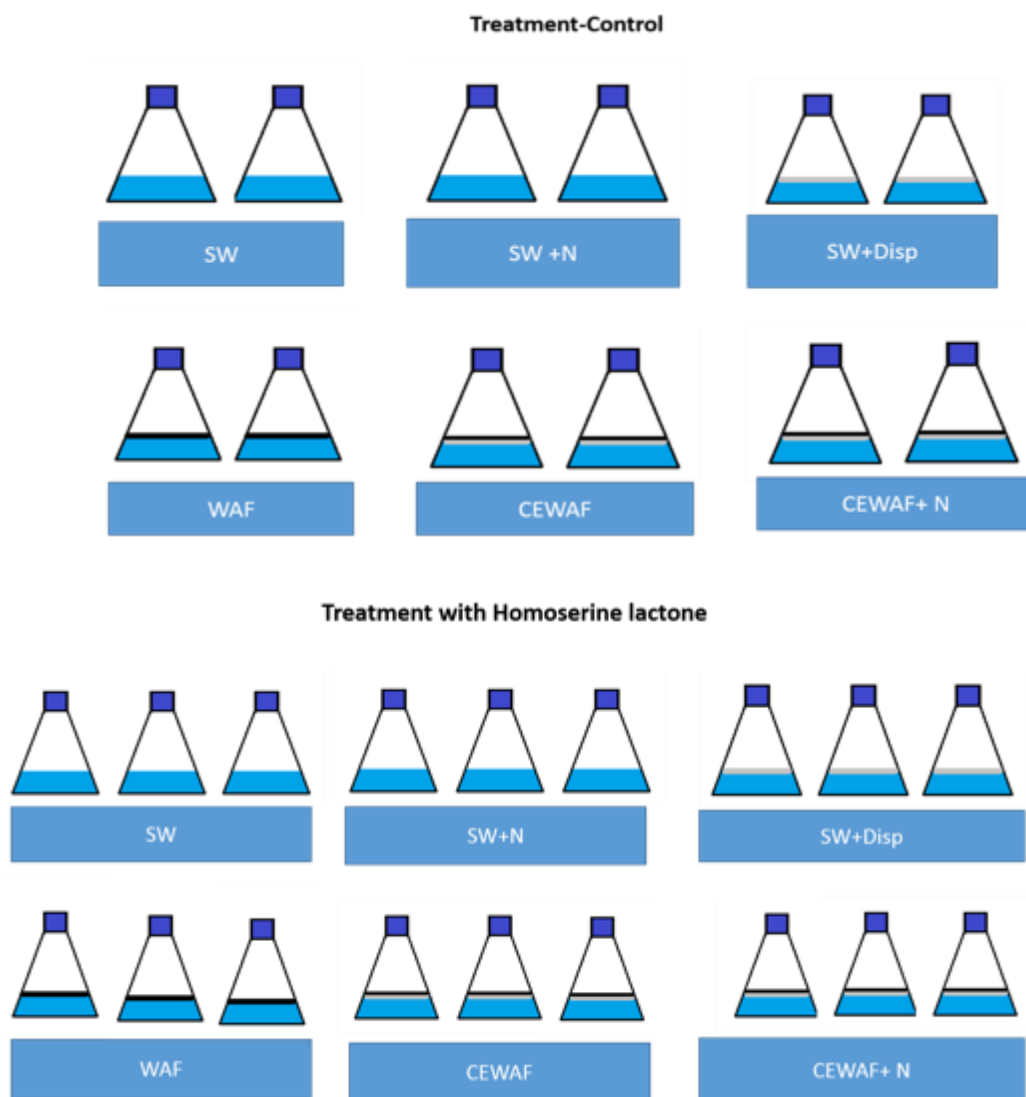
Fluctuations in behaviour of bacterial populations within the community, such as symbiosis, competence, virulence, extracellular enzymes, and biofilm formation are defined by the process of Quorum Sensing (QS) (De Kievit, 2009; Miller and Bassler, 2001). Therefore, it is important to assess the role of QS in the microbial community associated to marine oil snow and biofilms, and how this may influence the bioremediation of oil pollution in the ocean. Moreover, a previous study done by Gram *et al.* (2002) shows the potential presence and function of quorum sensing in marine snow aggregates. This experimental idea is to help further studies to assess the QS ability of marine bacteria communities in response to anthropogenic induced marine stressors – specifically crude oil. This work further improves the understanding of QS in marine microbial ecology.

## **2. Materials and Methods**

### **2.1. Sampling site and experimental set up**

This experiment was a reproduction of the one done in December 2015 (Chapter II of this thesis) to be able to see the effect of the addition of the chemical signal molecule – *L-Homoserine lactone* (described as involved in quorum sensing) to the seawater on MOS formation. The station FIM06a (60°38.12'N 4°54.03'W) was sampled at surface (5L from 3 m depth) the Faroe Shetland Channel, on May 2017 (same site than in Chapters II, III and IV). The seawater was stored in two different carboys at 4°C on board for one week and then transported to Heriot Watt University (Edinburgh, UK) where the experiments in the laboratory of the Life Sciences department. Due to the limited space on the roller table, the experiment was divided in two parts: Part 1 comprising four weeks of one set of control bottles and 1 concentration of *L-Homoserine lactone* (1:100) and Part 2 comprising one set of control bottles and another concentration of *L-Homoserine lactone* (1:10).

In a first step, water-accommodated fractions were done reproducing the experiment realised in December 2015 (Chapter II of this thesis). As a reminder, those fractions were elaborated such as the dispersant-only solution, WAF (Water Accommodated Fraction) and CEWAFs (chemically enhanced water-accommodated fraction), seawater was 0.22 µm filtered. WAF was prepared with 800 mL of sterile seawater amended with 140.8 mL filtered Schelallion crude oil. Dispersant-only solutions were comprised of 800 mL of sterile seawater and 14.08 mL of Superdispersant 25. CEWAFs were prepared with 800 mL of sterile seawater amended with 140.8 mL of filtered Schelallion crude oil and 14.08 mL of Superdispersant 25. Sterile seawater amended with oil and/or dispersant was mixed at 140rpm for 48 h at 7°C in the dark in sterile 500mL glass bottles. The fluid mixture was allowed to settle for 1h and the aqueous phase was sub-sampled into the tubes (autoclaved and acid washed) with Teflon caps, avoiding inclusion of the oil or dispersant phases.



**Figure D.1.** Scheme representing the experiment set up and the different number of treatments and corresponding replicates

In a first step, 2.4L of seawater was dispensed into clean and autoclaved 1L Pyrex glass bottles with teflon-lined caps. Roller tanks allow MOS to settle all the time without contact with tube surfaces (Daly *et al.*, 2016; Suja *et al.*, 2017). Then, 85.5mL of sterile WAF, dispersant-only, or CEWAF ( $\pm$ nutrients) was added to 300 mL of seawater. Microcosms were produced in duplicates for the controls and triplicates for the Homoserine lactone exposed bottles (see Figure D.1). They were kept at 10°C on a roller device in the dark at a rotation speed of 15 rpm. Five different time points ( $T_0$  after 0 days,  $T_1$  after 1 week,  $T_2$  after 2 1/2 weeks,  $T_4$  after 4 weeks and  $T_6$  after 6 weeks) were used to sub sample all the treatments. At each sampling time, samples were collected for DAPI cell counting, microscopic analysis. The treatments were daily observed for

possible MOS formation in all the treatments

## 2.2. Homoserine Lactone

Two different concentrations of *N*-(3-Oxododecanoyl)-L- homoserine lactone were studied here. In the first experiment, 5  $\mu$ M of *N*-(3-Oxododecanoyl)-L- homoserine lactone (QS) (Sigma-Aldrich, #O9139) and the second experiment 10  $\mu$ M (QSX) doubling the previous experiment concentration to see if the concentration of this molecule has a different effect on MOS formation. MOS formation was noted if observed and once the aggregates were big enough, they will be sampled and used for the next steps. Any aggregate from any kind of treatment were gently manipulated and collected for comparison between treatments.

### 2.2.1. Test of acylated homoserine lactone on MOS aggregates or other possible aggregates formed.

#### *Materials and Methods*

Preliminary screening for N-Acyl homoserine lactones (AHL) was done by preparing sterile filtered supernatants from cultures grown for 1.5 to 2 weeks at 15°C and testing the samples in three AHL monitor systems using *Agrobacterium tumefaciens* (Cha *et al.*, 1998) and *Chromobacterium violaceum* CV026 (McLean *et al.*, 1997) as described by Ravn *et al.* (2001). Since AHLs are not stable at high pH (above 8), all cultures should be grown in MB in which pH will be adjusted to 6.2. For the monitor assays, *A. tumefaciens* strain NT1(pZLR4) was grown with 20  $\mu$ g/ml<sup>-1</sup> of gentamicin in Luria-Bertani broth (Bertani, 1951) with 5 g of NaCl liter<sup>-1</sup> (LB5) for 24 h and inoculated into 50 ml of AB broth with 0.5% glucose and 0.5% Casamino Acids (Clark and Maaløe., 1967). The outgrown culture was mixed with 100 ml of melted, 45°C AB agar containing 50  $\mu$ g/ml<sup>-1</sup> of X-Gal (5-bromo-4-chloro-3-indolyl-u-Dgalactopyranoside) (Promega 9683801 L) and poured into petri dishes. *C. violaceum* CV026 is grown in LB5 with 20  $\mu$ g/ml<sup>-1</sup> of kanamycin for 24 h, inoculated in 50 ml of LB5, and incubated overnight. Plates were poured after the outgrown culture was mixed with 100 ml of 45°C LB5 agar.

Wells of 6 mm in diameter are punched in the solidified agars, and samples of 60  $\mu$ l are pipetted into the wells. Plates with *A. tumefaciens* or *C. violaceum* are incubated for 2 days and 1 day, respectively, at 25°C and read for zones of blue color due to AHL-induced u-galactosidase activity or zones of purple pigment due to AHL-induced violacein formation in the agar. MOS aggregates were then placed in those wells and incubated at 25°C and observed daily to see any change in colour around the aggregate.

### **2.2.2. Analyse gene expression to see how the gene in charge of AHL synthase is expressed**

*N-acyl-L-homoserine lactone* (AHL)-mediated gene expression is a cell density-dependent gene expression mechanism (Chong *et al.*, 2012). It involves the production of small membrane diffusible metabolites, AHLs, by an AHL synthase (LuxI homologue), interacting with their cognate receptor protein (LuxR homologue) when a threshold AHL concentration accumulates in the local environment thereby orchestrating gene expression (Manefield and Whiteley, 2007). Evidence has been generated suggesting that thin layers of cells can retard AHL diffusion (Mason *et al.*, 2005) and it is clear that AHL-mediated gene expression is active in biofilms harbouring AHL-producing bacteria (McLean *et al.*, 1997). *N-acyl-L-homoserine lactone*-mediated gene expression is encoded by three out of five classes of Proteobacteria (alpha-, beta- and gamma-), with approximately 7% of genera within these classes containing known AHL producing representatives (Manefield and Turner, 2002). In this study, it would be interesting to see if LuxI is expressed when MOS is formed and if its expression is different depending on the treatments.

### **2.2.3. Molecular detection of quorum sensing and chitinase genes**

This section was done with the help of Fenjgia Liu (Student of Prof. Theodore Henry at Heriot Watt University). RNA was extracted from the samples using the standard operating procedure for extracting total RNA for gene expression using phenol/chloroform extraction (Chomczynski and Sacchi, 2006; Chomczynski *et al.*, 2013; TRI Reagent SIGMA Technical Bulletin). After this, we measured the RNA concentrations extracted with a Nanodrop and it was realised that the concentrations of RNA were too low to keep working. We did several attempts in working through PCR and different dilutions to try to solve the problem but this was not possible. This same problem has been reported previously by other authors. This makes me suspect about the limitations of this method with the bacterial RNA.

This analysis could not be carried on due to low RNA concentration and lack of enough RNA material.

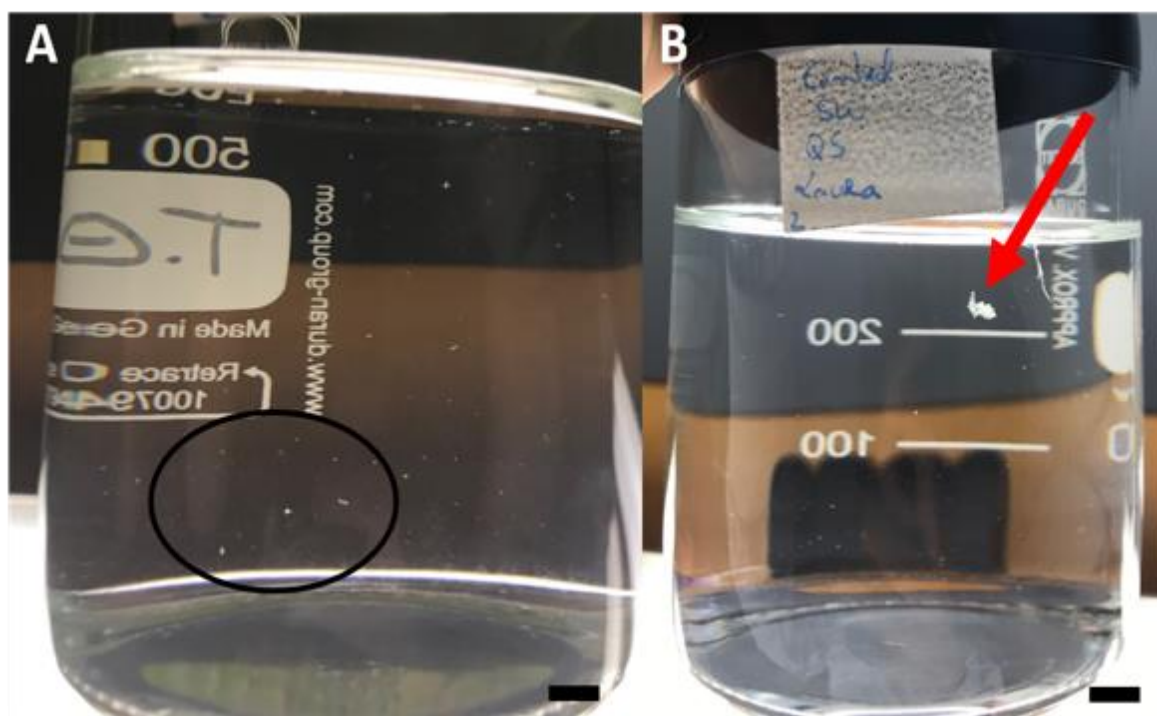
### *Zeta sizer*

The Zetasizer (nano series; Nano-ZS model, Malvern Panalytical) give the size of the aggregates that stay suspended in the water but not the ones that sink. 1mL of each sample was placed in a small cuvette for analysing it with the Zetasizer. The Zetasizer gives quite a lot of data such as Z average across time. Z average is an intensity-based overall average size based on a specific fit to the raw correlation function data. With the Zeta sizer, you also get the Zeta Potential or ZP (mV) that represents how much tendency the particles have to aggregate. If the value is between -30 and 30 that means that they will tend to aggregate. However, closer the value is to 0, higher is the tendency to bind and then form aggregates.

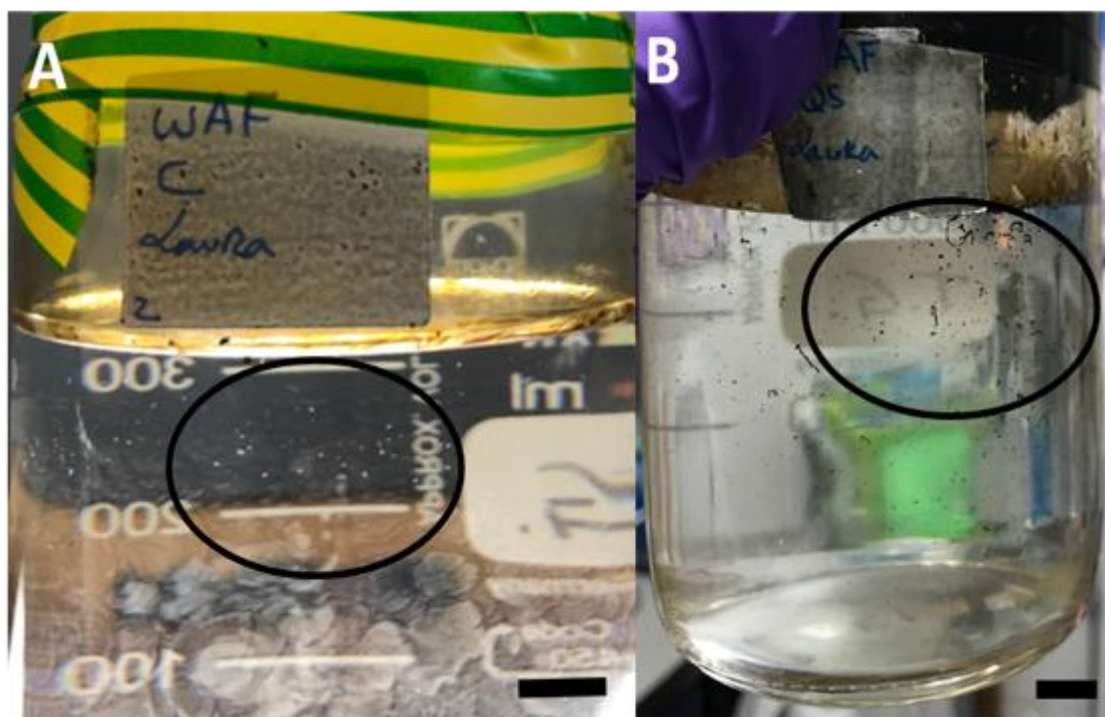
## **3. Results and Discussion**

### **3.1. MOS observation**

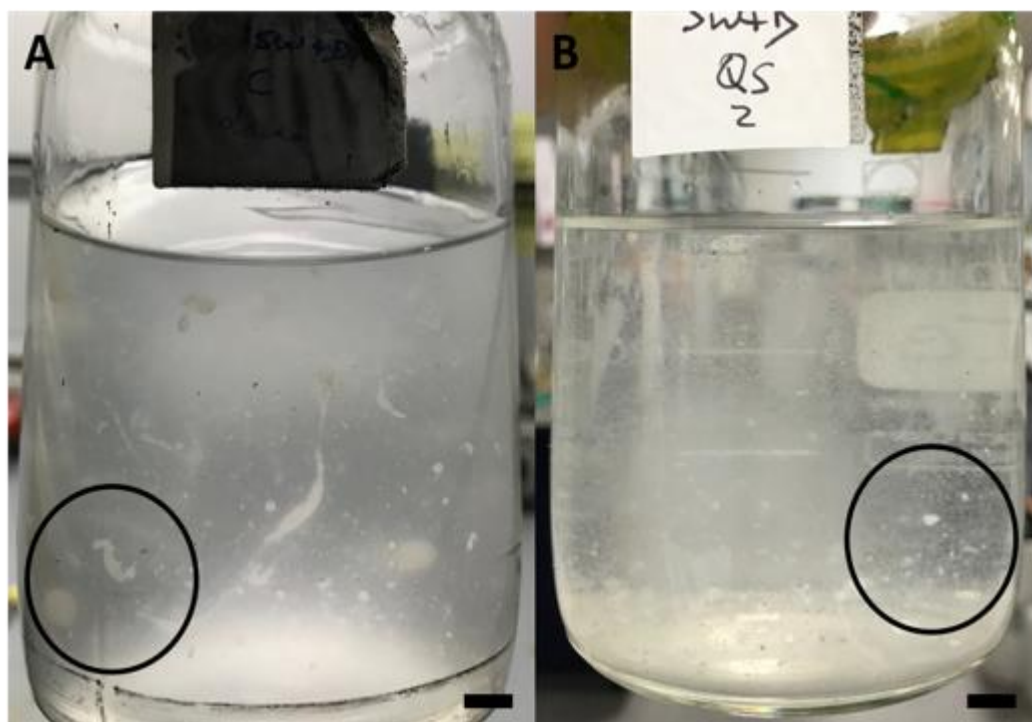
MOS seemed to be formed in different quantities and different shapes. In both cases, controls and AHL (QS) treatments, MOS aggregates were observed. In controls, MOS aggregates were in lower number but bigger while in AHL treatments MOS aggregates were in much higher numbers although smaller. All treatments showed aggregate formation: MS, MDS or MOS were respectively formed in SW/SW+N, SW+D and WAF/CEWAF/CEWAF+N. Aggregates formed after 4 days of experiments in all treatments. A wide range of aggregate size and shape was observed (from 0.01cmx0.01cm to 0.5cmx3cm). In all Figures D.2, D.3, D.4 and D.5, the wide range of size and shape of the different aggregates is reflected. Not all treatments nor timelines are represented due to too many pictures of high similarity, so the most relevant ones have been selected. Figure 2 shows the normal marine snow (MS) aggregates formed in the sea water under none treatment. This is used as a control to see if aggregates would form naturally without any kind of amendment to the sea water from the FSC. They indeed formed in both cases. In the control treatment (Figure 2A), aggregates were very small and fragile whereas in the treatment with QS (Figure 2B), aggregates were bigger and more solid. Figure 3 shows the MOS aggregates in WAF treatments. It is possible to see a difference in MOS aggregate shape and abundance under both conditions: control (Figure 3A) and under AHL treatment (Figure 3B). In Figure 3A, MOS looks very small and abundant of a light brown. However in Figure 3B, MOS looks more brownish and bigger.



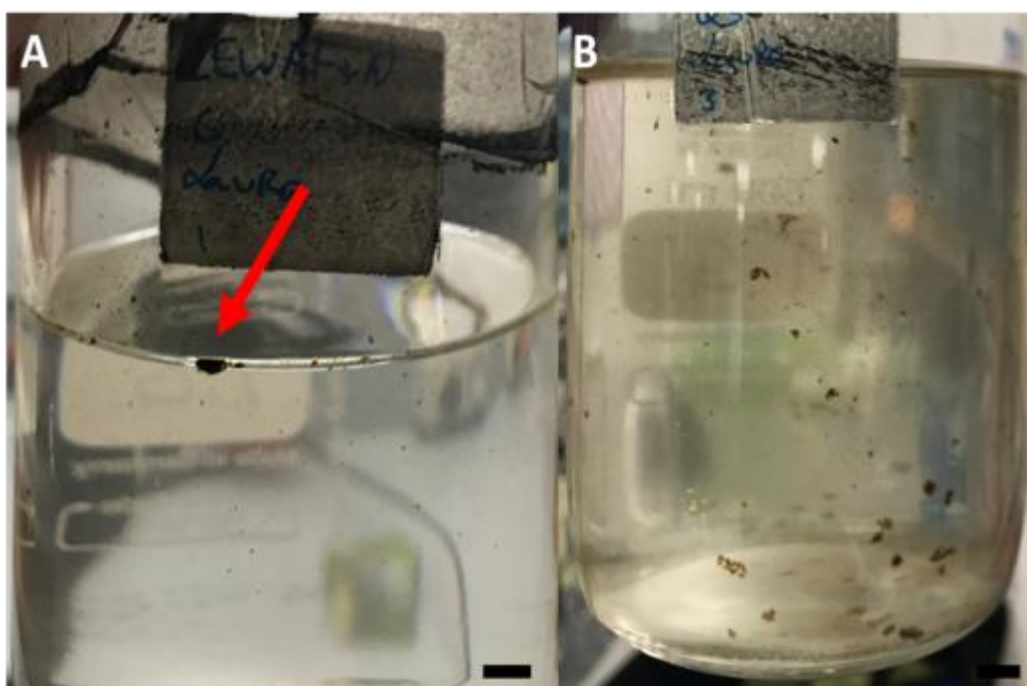
**Figure D.2.** MOS aggregates in the SW control treatments. A: Control, B: QS treatment. Scale bar: 1cm.



**Figure D.3.** MOS aggregates in the WAF treatments. A: Control, B: QS treatment. Scale bar: 1cm.



**Figure D.4.** MDS aggregates in the SW+D treatments. A: Control, B: QS treatment. Scale bar: 1cm.



**Figure D.5.** MS aggregates in the SW control bottles. A: Control, B: QS treatment. Scale bar: 1cm.

In the case of the Figure D.4, MDS aggregates in SW+D seem to be quite similar in both cases, in the control and in the treatment with AHL. Both cases showed indeed, an important number of MDS aggregates that look white, gluey and viscous. In Figure



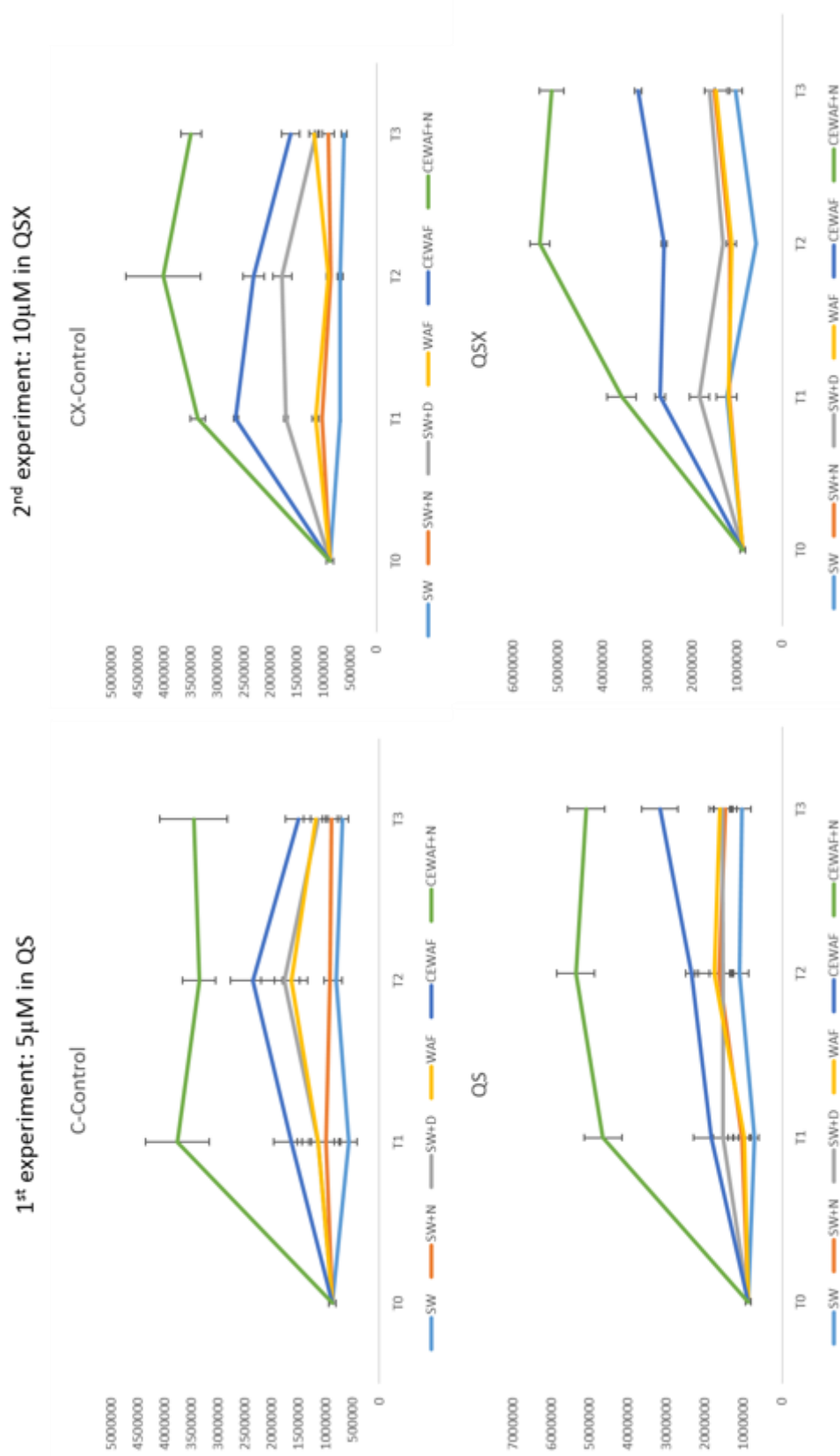
D.5, it is possible to see a difference in abundance of MOS aggregates in CEWAF+N treatments between the control (Figure D.5A) and the treatment under QS (Figure D.5B). It is possible to see that Figure D.5A has less and smaller aggregates than Figure D.5B. In both cases, MOS aggregates looked brownish and spongy as described in other studies (Kleindienst *et al.*, 2015a; Chapter II and III).

### 3.2. Cell count

Prokaryotic cells were counted at the different time points across all treatments of C (control) and QS (experiment amended with homoserine lactone). Aggregates were not sampled for cell counts. As it is possible to observe in Figure D.6, is that the number of bacteria is quite similar in both normal experiment and experiment under AHL exposure treatment. The treatment CEWAF+N in both cases shows the highest number of bacteria compared to the SW and SW+N (controls). Then, CEWAF+N seems to be the treatment with highest number of bacteria along the time in both cases. The addition of nutrients, oil and/or dispersant seems to stimulate the growth of the bacterial community. This has been observed in similar previous studies such as Kleindienst *et al.* (2015a) and Chapter II of this thesis.

The addition of AHL to the experiment does not seem to affect bacteria number or at least during the first 6 weeks of exposure. Maybe this experiment was not long enough to actually see an effect of AHL on the bacterial community dynamics. In the experiment ran under 5 $\mu$ M of AHL, the number of bacteria reaches the same number of bacteria than in control conditions (5x10<sup>6</sup> cells/mL).

There was no significant difference between time points (ANOVA,  $p=0.756>0.05$ ) nor treatment (ANOVA,  $p=0.944>0.05$ ). In the case of 10  $\mu$ M of AHL, the control will show the same range of number of bacteria than in the previous case. In the treatment with AHL, there was no significant difference between time points (ANOVA,  $p=0.844>0.05$ ) nor treatment (ANOVA,  $p=0.694>0.05$ ). It is possible to say then that the addition of AHL does not seem to have an effect on the total number of bacterial cells in the different treatments across the time.



**Figure D.6.** Number of bacteria per mL in the different treatments at different time points T0 (start of experiment), T<sub>1</sub> (week 1), T<sub>2</sub> (week 2) and T<sub>3</sub> (week 3).

### 3.3. Quorum sensing detection: Test of acylated homoserine lactone on MOS and MDS aggregates

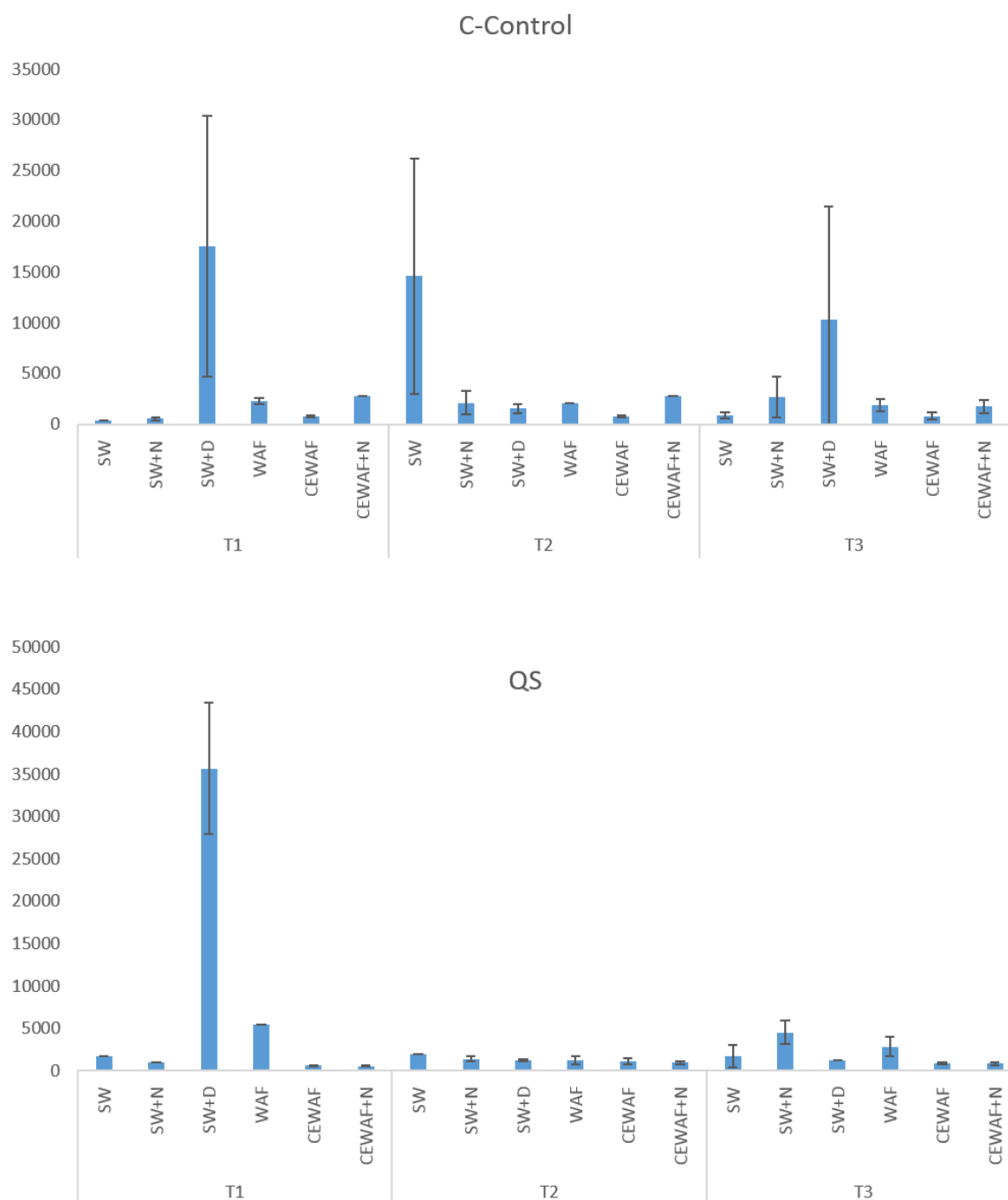
The bacteria grew well in the flasks and their corresponding media. However, this test did not work, not even the positive controls worked out. This could be due to the fact that something went wrong in the bacteria settlement on the agar or to the fact that the concentration of homoserine lactone by the bacteria present on the aggregates was not high enough to be detected. Also, maybe the homoserine lactone produced by those bacteria is not the one that would be recognised using the test. Anyway, these results are not the same than Gram *et al.* (2002) who detected positively AHL detection in marine snow aggregates. This need further work and investigation.

### 3.4. Aggregates size

Figure D.7 shows four graphs representing the differences in size of the aggregates or particles suspended in the water in the different treatments. It will only take in account the small suspended particles but not the big ones that sink to the bottom of the bottle. This method is then quite subjective since it discriminates a range of size of aggregates. It is possible to see that the treatment SW+D in C and QS experiments seems to show the highest values of average size of aggregates (around 2000-3000 d.nm). That makes sense since in the treatments with dispersant, the aggregates float much more and were quite abundant. Dispersant aggregates show a higher flotability in general than MOS maybe because the oil associated to MOS seems to make them heavier. It does not seem to be an important difference in aggregates suspended in QS or C treatments. That could be explained by the fact that a lot of aggregates were quite big and not taken in account by the Zeta sizer.

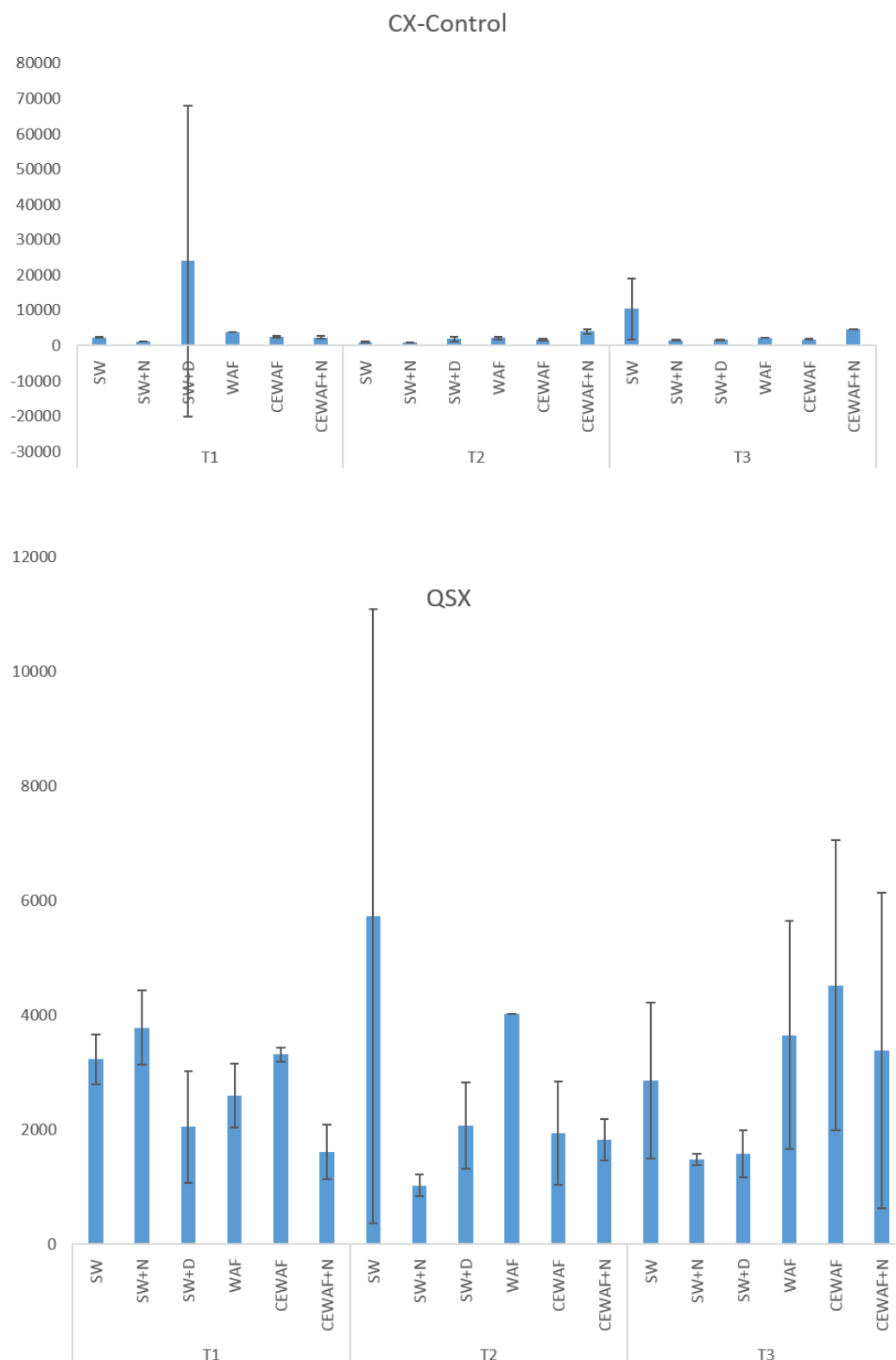
In Figure D.7, the experiment under 5 $\mu$ M of AHL, there is generally a no significant difference between the control experiment and the AHL experiment (ANOVA,  $p=0.149>0.05$ ) across treatments. This means that the concentration of 5 $\mu$ M of AHL does not seem to have any effect on aggregate size across treatments, replicates nor time compared to the control. In Figure D.8, the second experiment 10 $\mu$ M of AHL, there is a general significant difference between CX and QSX (ANOVA,  $p=0.022<0.05$ ) but not across treatments in CX (ANOVA,  $p=0.062$ ) and QSX (ANOVA,  $p=0.138>0.05$ ). There were not significant differences either between time points in CX (ANOVA,  $p=0.141>0.05$ ) and QSX (ANOVA,  $p=0.065>0.05$ ).

# 1<sup>st</sup> experiment: 5 $\mu$ M in QS



**Figure D.7.** Average size of aggregates (d.nm) and its corresponding standard deviation given by Zetasizer for both experiments: Control and QS (under AHL treatment).

## 2<sup>nd</sup> experiment: 10 $\mu$ M in QSX



**Figure D.8.** Average size of aggregates (d.nm) and its corresponding standard deviation given by Zetasizer for both experiments: Control and QS (under AHL treatment).

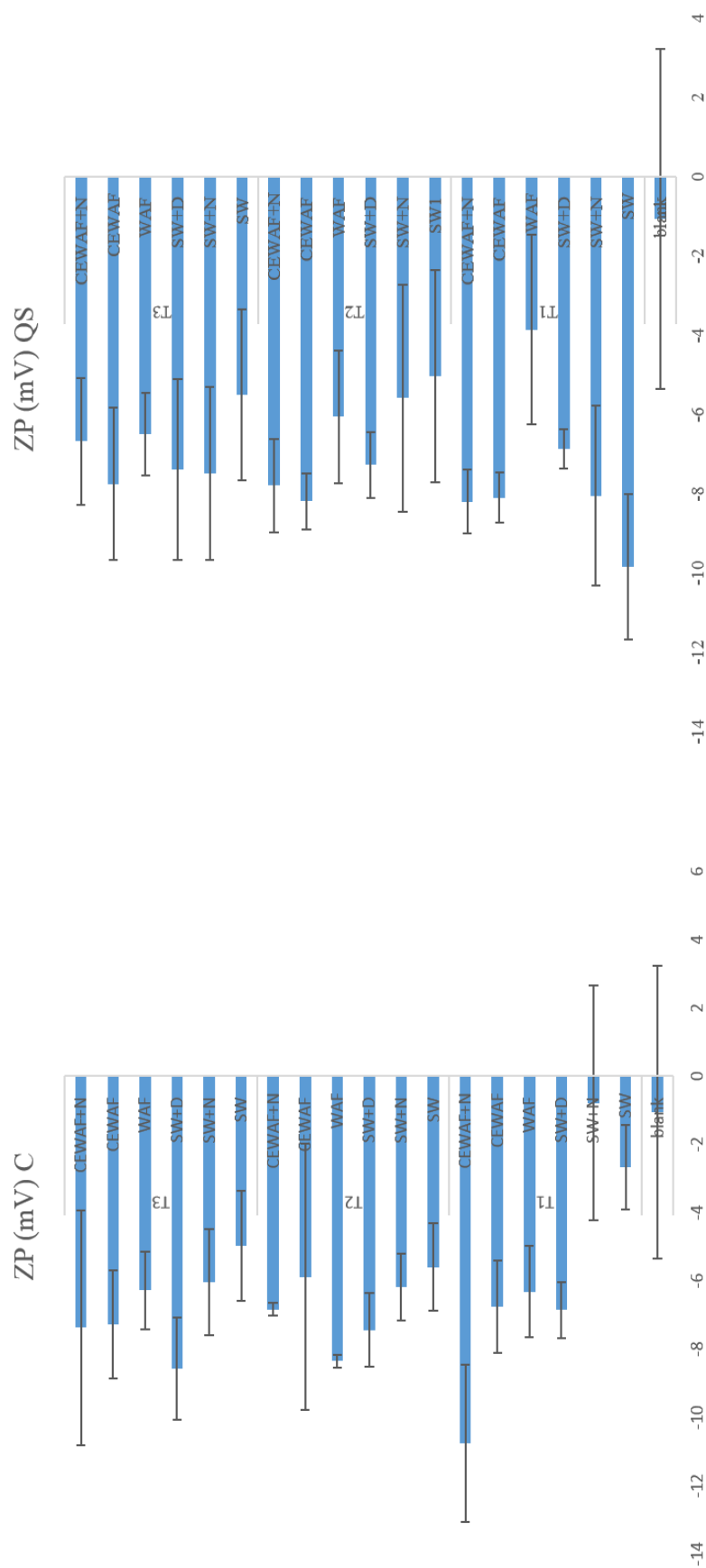
This means that in this case, AHL might have a significant effect on aggregate size. However, those results should not be really trusted by the fact that this method discriminates big aggregates that are not suspended anymore.

### *Tendency of aggregation*

As said previously, Zeta potential is described as the key indicator of how likely are the suspended particles likely to aggregate. In Figure D.9, the results of this analysis of the experiment with 5 $\mu$ M of AHL are represented. It is possible to see that in both cases, Control and under 5 $\mu$ M AHL concentration, there is similar values although they seem slightly higher in the case of AHL. All values are between -30mV and 30mV and quite close to 0, so this means that generally the particles suspended in the seawater of our experiment, have already a high tendency of aggregation. This technique has the same uncertainties than for the aggregate size. It only calculates the potential of aggregation of the suspended particles, so the particles that have already aggregated or are too big to be suspended in the seawater, are not taken in account. That is why the results of this technique should not be completely trusted and it would be beneficial for further studies to use another technique and compare the results with this one.

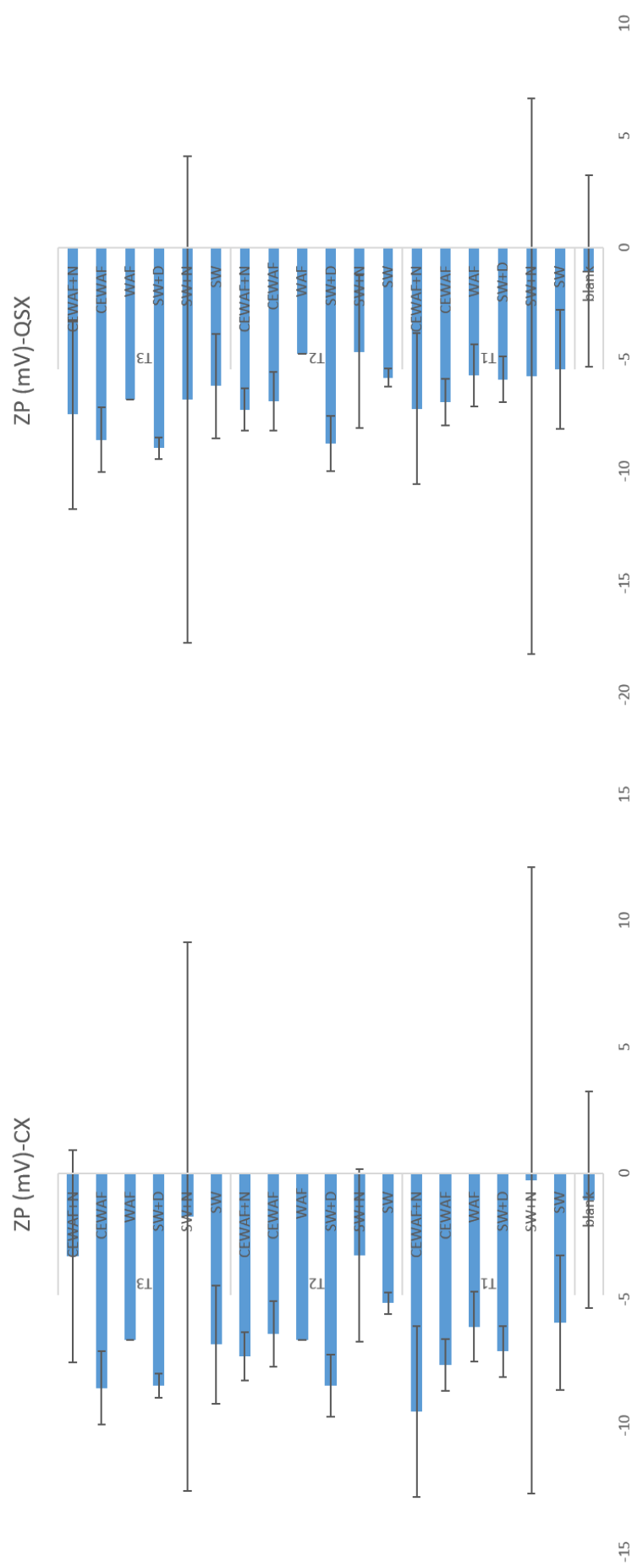
In Figure D.9, there is a general no significant difference across the control and AHL exposed experiments under 5 $\mu$ M treatment (ANOVA,  $p=0.098>0.05$ ). This means that there are not differences across treatments nor timeline nor exposure (C or QS) in this case. The addition of AHL does not seem to influence the Zeta potential of the suspended particles in the water. Same happens in Figure D.10 with 10 $\mu$ M QSX, values do not seem to be different from the ones observed in the 5 $\mu$ M QS experiment. This means that the concentration of AHL does not seem to have an influence on particle aggregation. In Figure D.10, there is a general no significant difference across the control and QSX experiments under 10 $\mu$ M treatment (ANOVA,  $p=0.127>0.05$ ). This means that there are not differences across treatments nor timeline nor exposure (CX or QSX) in this case. The addition of AHL does not seem to influence the Zeta potential of the suspended particles in the water.

# 1<sup>st</sup> experiment: 5μM in QS



**Figure D.9.** Average of the potential of particles to aggregate (mV) across the time in all treatments with their corresponding standard deviation.

## 2<sup>nd</sup> experiment: 10 $\mu$ M in QSX



**Figure D.10.** Average of the potential of particles to aggregate (mV) across the time in all treatments with their corresponding standard deviation.



In summary, the amendment of AHL molecule signalling independently of its concentration, does not seem to alter nor modify the tendency of aggregates to form. This is the first study reporting and measuring the tendency of particles suspended on sea water to form aggregates with or without addition of dispersant and/or oil. The use of the Zetasizer method is quite subjective because it discriminates the big aggregates that form and sink straight away. It would be interesting to use other methods and compare results to see whether it is possible to confirm these results or not.

#### **4. Conclusion**

Bacterial community numbers seem to follow the pattern of other studies such as Chapter II of this thesis where the highest number of cells per mL is in the treatment CEWAF+N. Moreover, the amendment of the treatments by the highest concentration of AHL (10 $\mu$ M) shows a significant in aggregate size compared to the control. This could suggest, that a high concentration of AHL could enhance the size of the MOS aggregates. In this study, preliminary studies to investigate if the Quorum Sensing is a potential factor for MOS formation are reported. It is the first time that these methods are used for the study of aggregates. Those methods seem to have positive and negative aspects: it is possible to detect and measure the size suspended particles in the water and their tendency to aggregate. However, the big aggregates that do not stay suspended are discriminated, so some information could be lost.

Further studies and the use of other methods could be useful for a better understanding of the role of the AHL in relation to the bacteria and the MOS formation. This research could be in the pathway for the future as a potential way of bioremediation to an oil spill.

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